Tumor Associated Macrophages Regulate Epithelial to Mesenchymal Transition in Tumor Cells in a TGF-β dependent Manner

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1. Zusammenfassung

Das Wachstum eines Tumors hängt von einer Reihe von limitierenden Schritten ab, die in höchstem Masse von der Mikroumgebung des Tumors beeinflusst werden. In diesem Zusammenhang gelten Tumor-assoziierte Makrophagen (TAM) als hauptsächliche Regulatoren des Tumorwachstums; dies einerseits wegen deren Einfluss auf die Angiogenese, auf den sogenannten „angiogenic switch“, und andererseits wegen deren vielfältigen Funktionen im Modellieren der Tumormikroumgebung. Diese Ereignisse erleichtern und orchestrieren die Invasion und Verbreitung von Tumorzellen ins benachbarte Gewebe, weshalb man auch begünstigende Effekte der TAM auf die Bildung von Metastasen vermutet. Ihr genauer Einfluss in diesen komplexen Prozessen ist allerdings noch nicht vollständig aufgeklärt.

Schon früher haben wir und andere die vielseitige Rolle von TAM im Tumorwachstum studiert, indem wir TAM in Tumor tragenden Mäusen mit einer pharmakologischen Methode depletierten. Diese Studien haben erfolgreich eine kausale Verbindung zwischen TAM Depletion und reduziertem Tumorwachstum hergestellt, wobei die TAM-abhängige Tumorangiogenese als hauptsächlicher Mechanismus identifiziert wurde. In der präsentierten Arbeit habe ich diese auf Bisphosphonat-Liposomen basierte Technik der TAM Depletion im F9-Terato-karzinom Mausmodell angewendet, und mit Hilfe einer Genexpressionsanalyse dieser Tumore Genprofile identifiziert, welche zusätzliche und neuartige Rollen für TAM in Tumorwachstum und -entwicklung aufdeckten.

Resultate dieser Analyse haben eine Korrelation zwischen TAM-Dichte und einem Genprofil ergeben, das der sogenannten epithelial-mesenchymalen-Transition (EMT) entspricht. EMT ist ein gut charakterisierter Mechanismus, durch welchen epithelartige Tumorzellen einen invasiven mesenchymalen Phänotyp erlangen können, der als limitierender Schritt in der Tumorinvasion und Metastasierung betrachtet wird. Die gefundene Korrelation zwischen TAM-Dichte und EMT-assoziiertem Genprofil suggeriert demzufolge eine Rolle der TAM in der Regulation der EMT-assoziierten Tumorinvasion. Dieser Befund wurde weiter in Zellkulturen von F9- und NMuMG-Zellen und mit molekularen und zellbiologischen Techniken untersucht. Dabei wurde der TAM-abhängige Faktor TGF-β1, und die durch diesen Faktor induzierte Aktivierung der β-Catenin Signalkaskade, als Hauptmechanismus der Tumorzell-EMT identifiziert.

Die TAM-induzierte EMT führte zu erhöhter Tumorzellinvasion als Antwort auf Makrophagen abhängige Chemokine. Die Invasion wurde jedoch nach Neutralisierung von TGF-β1 wieder aufgehoben. TGF-β1 alleine erwies sich nicht als genügend starkes Chemoattraktans für die untersuchten Zelllinien. Demzufolge zeigen die Daten einen direkten Einfluss der TAM auf die intrinsische Regulierung invasiver Eigenschaften von Tumorzellen. TAM sind eine wichtige Quelle verschiedenster Faktoren, wie z.B. SDF-1, VEGF und EGF und die Ergebnisse suggerieren demnach ein Modell in welchem TAM durch eine TGF-β abhängige Signalübertragung einen invasiven Tumorzellphänotyp via EMT ausbilden und gleichzeitig durch Signalübertragung von Chemokinen den Tumorzellen wachstums-gerichtete Stimuli bieten.

Ein wichtiger Aspekt dieser Studie war die Evaluation der klinischen Relevanz der TAM-induzierten Tumor-EMT im Verlauf einer Tumorerkrankung. Diese Relevanz wurde durch eine histopathologische Analyse von 491 Tumorproben von nicht kleinzelligen Lungenkarzinomen („non-small cell lung cancer“, NSCLC) bestätigt, wobei eine signifikante Korrelation zwischen intra-tumoraler Makrophagendichte, mesenchymalem Tumorzell-Phänotyp und dem Entwicklungsgrad der Tumore gezeigt werden konnte. Diese klinische Relevanz der grundlegenden *in vivo* und *in vitro* Ergebnisse weist darauf hin, dass therapeutische Ansätze gegen TAM den Krankheitsverlauf von EMT-assoziierten Tumorformen möglicherweise verbessern könnten.

Insgesamt kann durch die Resultate aus meiner Doktorarbeit eine neuartige Dimension im komplexen Zusammenspiel von Tumorzellen und der Tumor-mikroumgebung erkannt werden.

1. Summary

Tumor progression depends on a number of rate-limiting steps that are highly influenced by the tumor microenvironment. To this end, tumor associated macrophages (TAMs) are regarded as key regulators of tumor progression. This is in part due to their involvement in the “angiogenic switch” and their functions in modulating tumor growth. Moreover, TAMs secrete a vast amount of chemokines through which they attract motile cancer cells. These events facilitate and orchestrate tumor cell invasion and dissemination, thus TAMs are generally believed to promote tumorigenesis and metastatic disease. However, their exact involvement in these processes and the mechanisms of action remain elusive.

Our laboratory and others have previously studied the versatile roles of TAMs in tumor progression by performing pharmacologic depletion of TAMs in tumor bearing mice. These studies successfully linked TAM-depletion to reduced tumor growth and identified TAM-dependent tumor-angiogenesis as a major regulator hereof. In the presented work, I have used this bisphosphonate-liposome based technique to deplete TAMs in F9-teratocarcinoma tumors. I have combined this technique with gene expression analysis performed on the isolated F9-tumors and identified gene expression profiles revealing additional and novel implications for TAMs in tumor growth and progression.

Data obtained from this comparative gene expression analysis revealed a correlation between TAM density and a gene expression profile characteristic of epithelial to mesenchymal transition (EMT). EMT is a well characterized mechanism through which epithelial tumor cells can acquire an invasive, mesenchymal phenotype, and it is therefore regarded to be a rate-limiting step in tumor invasion and progression. Thus, the correlation between TAM density and

EMT-associated gene expression suggested a role for TAMs in regulation of EMT-associated tumor invasion. This was confirmed in cell cultures of murine F9- and mammary gland NMuMG-cells by using molecular and cell biological techniques. Importantly, TAM-derived TGF-β and consecutive activation of the β-catenin pathway was identified as a main inducer of tumor cell EMT.

TAM-induced EMT led to increased invasion in response to macrophage-derived chemokines, however invasion was abrogated upon neutralization of TGF-β1. TGF-β1 alone did not prove to be a potent chemoattractant for the tested cell lines, thus the data suggests that TGF-β regulates tumor cell invasion by induction of a chemotactic phenotype, rather than providing chemoattracting signaling. The data demonstrate a direct involvement of TAMs in the intrinsic regulation of invasive properties in tumor cells.

An important aspect of this study was to evaluate the clinical relevance of TAM-induced tumor EMT in disease progression. This was done by a histo-pathologic analysis of 491 non-small cell lung cancer specimens, which revealed a significant correlation between intra-tumoral macrophage density, mesenchymal tumor cell phenotype and tumor grade. This underscores the clinical relevance of the murine data and suggests that therapeutic targeting of TAMs may improve management of cancers susceptible to stroma induced EMT-associated disease progression.

Collectively, the data obtained during the course of my PhD unravel a novel dimension in the complex interplay between tumor cells and the tumor stroma and provide novel insight valuable for the development of therapies abrogating EMT-associated disease progression.

2 Introduction

After several decades of rapid advances in the development of cancer therapies, the disease remains the second most frequent cause of death in the western world. Cancer is essentially a genetic disease, although lifestyle and environmental factors are known to contribute, and for two decades the major research topic has been the identification and characterization of genetic alterations causing neoplastic cell transformation (Murga and Fernandez-Capetillo, 2007). This has opened a window of therapeutic opportunities, targeting and eradicating cells with compromised integrity. However, the success of these therapies has been limited, suggesting that an important aspect of tumor pathogenesis has remained unresolved.

Significant achievements made during the last two decades have led to the concept of “hallmarks of cancer”. These hallmarks are generally defined as alterations in gene expression leading to self sufficiency in cell growth, insensitivity to growth inhibiting signaling, limitless replication potential, evasion of apoptosis, acquisition of invasive properties and neo-angiogenesis (Fig. 1), (Hanahan and Weinberg, 2000). However, neo-angiogenesis has been found to be tightly regulated by extrinsic factors hosted by the tumor microenvironment (Ingber and Folkman, 1989, Hartlapp *et al*. 2001, Lin *et al*. 2006, Joyce and Pollard, 2009). The notion that the tumor microenvironment can be rate-limiting for tumor progression was first proposed by Stephen Paget in his “seed and soil” theory from 1889 (Ribatti *et al*. 2006). Paget postulated that a metastatic tumor is established only when the tumor cells and the host environment are compatible. A century later, experimental implantation of murine cancer cells in non-tumorigenic host environments confirmed that the malignant potential of neoplastic cells highly

depends on the nature of the host tissue (Mintz *et al*. 1975, Dolberg and Bissel, 1984).

Although these studies manifested the importance of the microenvironment in tumorigenesis, it is only during the past 15 years that we have begun to understand the underlying molecular mechanisms. Intensive investigations of these mechanisms have revealed a highly complex pattern of signal transducing pathways tightly regulated by the tumor stroma. Of the signaling pathways identified to date, a significant number are associated with wound healing and inflammation, and this has revived another century old paradigm stating that tumors are never healing wounds (Dvorak, 1986, Coussens and Werb, 2002, Grivennikov *et al*. 2010). This has led Mantovani and co-workers to propose tumor inflammation as the seventh "hallmark of cancer", thereby highlighting the importance of the host environment in disease progression and concurrently drawing attention to paradigms made more than a century ago (Fig. 1), (Colotta *et al*. 2009).

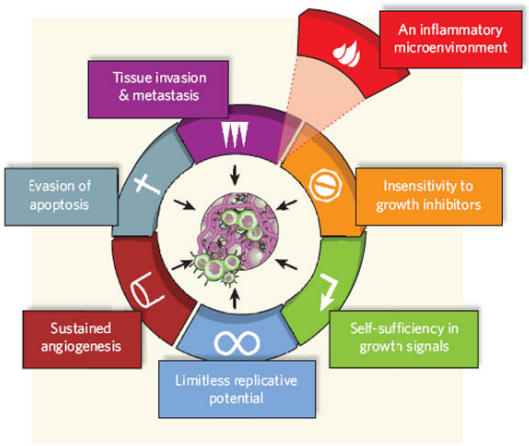

Fig. 1. Schematic illustration of the seven hallmarks of cancer. Adapted from Colotta *et al*. 2009.

The recognition that tumor progression is influenced by the microenvironment opens a new window of therapeutic opportunities. However, the success of therapies targeting the tumor stroma highly depends on the identification and thorough examination of key regulators hosted in the microenvironment. The following sections review our current knowledge of the tumor promoting properties of the microenvironment, and special attention will be given to tumor associated macrophages.

2.1. Hosting a tumor; the tumor microenvironment

Solid tumors are complex organ-like structures comprised of tumor cells, stromal cells and extracellular matrix (ECM). Although transformed epithelial cells represent the only malignant cell type, the cellular composition of the microenvironment, and the adapted behavior of infiltrated stromal cells, contributes significantly to the malignant potential of a tumor (Tse and Kalluri, 2007, Joyce and Pollard, 2009). Tumor cells recruit a vast amount of leucocytes, such as neutrophils, natural killer cells (NKs), macrophages, dendritic cells and T-cells, as well as mast cells and fibroblasts through extensive chemokine signaling (Bellocq *et al*. 1998, Wyckoff *et al*. 2004, Cozar *et al*. 2005, Parsonage *et al*. 2005, Kalluri and Zeisberg, 2006, Green *et al*. 2009, Joyce and Pollard, 2009, Tazzyman *et al*. 2009). Upon tumor infiltration, these cells initiate a pro-inflammatory response similar to that known from wound healing (Dvorak, 1986, Coussens and Werb, 2002).

A central regulator of this pro-inflammatory response is the transcription factor nuclear factor-κB (NFκB), which is activated e.g. by tumor necrosis factor-α (TNF-α) and lipo-polyssaccharide (LPS) mediated Toll-like receptor signaling (Tak and

Firestein, 2001, Lu *et al*. 2006, Wu and Zhou, 2010). Under normal homeostatic conditions, NFκB is sequestered in part by the adherence junction complex, where it associates with E-cadherin and β-catenin, and to a greater extent by its molecular inhibitor IκB (Novak *et al*. 1991, Lu *et al*. 2006, Solonas *et al*. 2008). NFκB is activated upon e.g. TNF-α mediated phosphorylation and following degradation of IκB or by phosphorylation and following dissociation of the adherence junction complex. The active NFκB translocates to the nucleus to initiate transcription of target genes, which includes various pro-inflammatory cytokines, mitogens and self regulating genes (Lu *et al*. 2006, Wu and Zhou, 2010). The acute pro-inflammatory response is the initiating event in wound healing. It is followed by the "proliferative phase", which is characterized by recruitment and proliferation of endothelial cells and capillary support cells for angiogensis, and recruitment of proliferating epithelial cells necessary for successful re-epithelialization. The final step in wound healing is tissue remodeling, which is mainly facilitated by infiltrated myofibroblasts, which contribute to this process by extensive secretion of matrix proteins. Moreover, myofibroblasts physically close the wound by docking on opposite tissue boundaries and via cellular contractions bring the wound margins together (Grinnell, 2000, Coussens and Werb, 2002). Upon wound closing, the pro-inflammatory cytokines are substituted by anti-inflammatory cytokines consequently inducing apoptosis in the redundant infiltrates and the inflammation resolves (Maiuri *et al*. 2004, Lu *et al*. 2006). However, whereas the wound healing process is self-limited in part due to the self regulating loop of NFκB, the pro-inflammatory response in tumors often persists and becomes chronic (Coussens and Werb, 2002, Lu *et al*. 2006).

As mentioned, due to the similarities is cellular composition, signaling and biology, solid tumors are often referred to as “never healing wounds” (Fig.2), (Dvorak, 1986, Coussens and Werb, 2002)

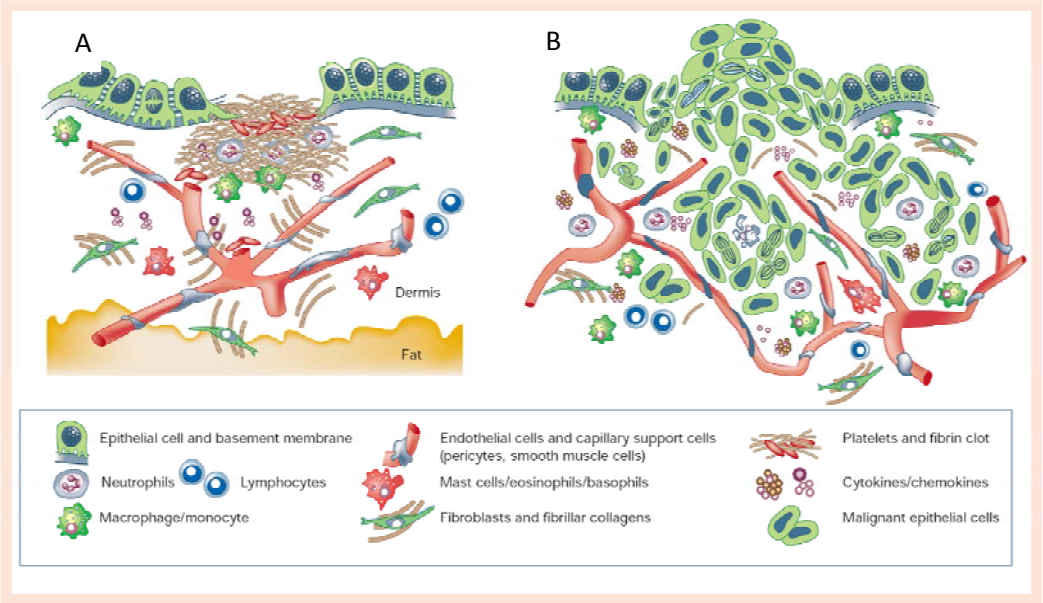

Fig. 2. Schematic illustration of wound healing and tumor growth. A. Non pathologic tissues are highly organized structures with epithelial cells resting on the basement membrane. Upon rupture, the wound is temporarily closed by platelets and fibrin cloths and leucocytes and myofibroblasts are recruited to the site where they incite an acute pro-inflammatory response to mediate angiogenesis and epithelialization to close the wound. **B**. Invasive carcinomas are highly unorganized. Epithelial cells lose attachment to neighboring cells and to the basement membrane as they become motile and neo-angiogenesis is randomly induced where neoplastic cells interact with remodeled ECM, inflammatory cells or myofibroblasts. The high production of pro-inflammatory cytokines by tumor cells and inflammatory cells stimulates chronic inflammation and the production of matrix proteins and proteinases which, besides ECM remodeling, permeabilize the tumor vasculature and make them accessible for migratory tumor cells, thereby facilitating tumor dissemination and metastasis. Adapted from Coussens and Werb, 2002.

2.1.1 Cancer associated fibroblasts and the ECM

Myofibroblasts are mesenchymal cells derived from bone marrow precursors and they are recruited to sites of inflammation or tumors by epithelial cell derived chemokine gradients (Parsonage *et al*. 2005, Kalluri and Zeisberg, 2006). Although traditionally regarded to be of mesenchymal stem cell origin, emerging evidence suggests that a large fraction of fibroblasts found in the tumor stroma, the cancer associated fibroblasts (CAFs), may originate from trans-differentiated

epithelial tumor cells (see section 2.4), (Iwano M. *et al*. 2002, Kurose *et al*. 2002, Franci *et al*. 2006, Radisky *et al*. 2007, Tse and Kalluri, 2007).

Trans-differentiation can occur as a consequence of epithelial to mesenchymal transition (EMT), a cellular mechanism through which e.g. tumor cells lose their epithelial traits and acquire mesenchymal properties (Thiery, 2002, Nawshad *et al*. 2005, Polyak and Weinberg, 2009). EMT is crucial for embryogenesis and tissue repair, and although it is traditionally regarded to facilitate cell migration, it may in the case of wound healing, represent a mechanism to rapidly supply an injured tissue with fibroblasts to mediate the repair (Thiery, 2002, Thiery *et al*. 2009).

CAFs main activity in tumors is, as in the case of wound healing, remodeling of the ECM. They contribute dual sided to this process as they secrete proteinases for matrix breakdown, and simultaneously deposit matrix proteins directly into the matrix (Rodemann and Müller, 1991, Kessenbrock *et al*. 2010). Due to chronic inflammation, the production of matrix proteins often exceeds the local requirements which can result in desmoplasia, ultimately leading to mechanical invasion of the surrounding tissue (Coussens and Werb, 2002, Kalluri and Zeisberg, 2006).

The ECM comprises proteoglycans, collagens and fibronectin, which fill out the interstitial spaces and provide a site of anchorage for migrating cells (Kessenbrock *et al*. 2010). Moreover, it is rich on laminins which are the main components of the basement membrane (Kalluri and Zeisberg, 2006). Besides supporting the tumor with connective tissue, the ECM plays an important role in cell signaling. It sequesters large amounts of cytokines and growth factors which are activated upon proteolytic cleavage of the matrix. It thus allows rapid and local growth factor mediated activation of cellular processes creating a dynamic tumor microenvironment with an obvious growth advantage (Kessenbrock *et al*. 2010).

In addition to these rather indirect implications in tumor progression, CAFs also contribute directly to the “hallmarks of cancer” through cytokine and growth factor signaling. To this end, they stimulate tumor cell proliferation through secretion of soluble growth factors and mitogens and they contribute to tumor angiogenesis by vascular endothelial growth factor (VEGF) and stromal derived factor-1 (SDF-1/CXCL12) mediated recruitment and proliferation of endothelial cells (Hlatky *et al*. 1994, Orimo *et al.* 2005, Kalluri and Zeisberg, 2006, Studebaker *et al*. 2008, Erez *et al*. 2010). Both SDF-1 and VEGF are potent chemoattractants for leucocytes, and CAFs may thus contribute significantly to chronic tumor inflammation (Leek *et al*. 2000, Orimo *et al*. 2005, Parsonage *et al*. 2005). CAFs implication in sustained tumor inflammation is further substantiated by a recent report in which gene profiling identified constitutive activation of the pro-inflammatory NF-κB pathway in CAFs. Thus, cancer associated fibroblasts play a significant and versatile role in tumor progression, and as a consequence they represent a strong therapeutic target (Kalluri and Zeisberg, 2006, Tse and Kalluri, 2007, Erez *et al*. 2010)

2.1.2 Tumor infiltrating leucocytes

Tumor infiltrating leucocytes constitute approximately 50% of solid tumors and are thus the main component of the tumor stroma (Sica *et al*. 2008a). This observation has traditionally been interpreted as an immune response against the tumor, but tumor immunology studies performed over the past decade has revealed a common cancer related shift in the classical immune response in favor of tumor growth (DeNardo and Coussens, 2007, Sica *et al*. 2008a).

The polarization of leucocytes is determining for whether the immune system mounts a anti-tumorigenic or pro-tumorigenic response. In both cases the initial

event is and acute pro-inflammatory immune response mounted by innate immune cells, mainly by secretory myeloid derived cells (see section 2.2), natural killer cells (NKs) and mast cells (DeNardo and Coussens, 2007, Sica *et al*. 2008a, Grivennikov *et al*. 2010). These pro-inflammatory cells directly eliminate pathogens through cytotoxic responses and phagocytosis. Moreover they secrete a variety of cytokines and chemokines to recruit additional leucocytes, mainly lymphocytes of the adaptive immune system (DeNardo and Coussens, 2007, Grivennikov *et al*. 2010). Professional antigen presenting cells (APCs), e.g. dendritic cells and macrophages, play a crucial role in mounting the adaptive immune response, as they take up foreign antigens and present them to T- and B-cells in the sentinel lymph nodes (DeNardo and Coussens, 2007). Successfully primed lymphocytes, $CD4^+$ Th-cells and their cytotoxic $CD8^+$ counterparts, subsequently infiltrate the damaged tissue and mount a cytotoxic response to eradicate pathogens (Th_1 response). After successful eradication, the cytokine profile in the microenvironment changes to stimulate an anti-inflammatory reponse, which recruits $CD25^+$ regulatory T-cells ($CD25^+T_{regs}$) to induce immune tolerance and resolve the inflammation, leading to tumor rejection (Erdman *et al*. 2005, Porcheray *et al*. 2005, DeNardo and Coussens, 2007, Grivennikov *et al*. 2010), (Fig. 3).

However, when the anti-inflammatory response fails, the inflammation becomes chronic. Suppression of the anti-inflammatory response is promoted by tumor derived IL-4, IL-5, IL-6, IL-10 and IL-13, and as a consequence $CD4^+$ T-cells fail to activate $CD8^+$ T-cells, and thereby the tumor effectively evades a cytotoxic response. Moreover, activated B-cells in cooperation with dendritic cells, contribute to immune evasion by converting naïve Th-cells into $CD4^+CD25^+T_{regs}$

further stimulating immune tolerance (Th_2 response), (DeNardo and Coussens, 2007, Moore *et al*. 2010, Grivennikov *et al.* 2010).

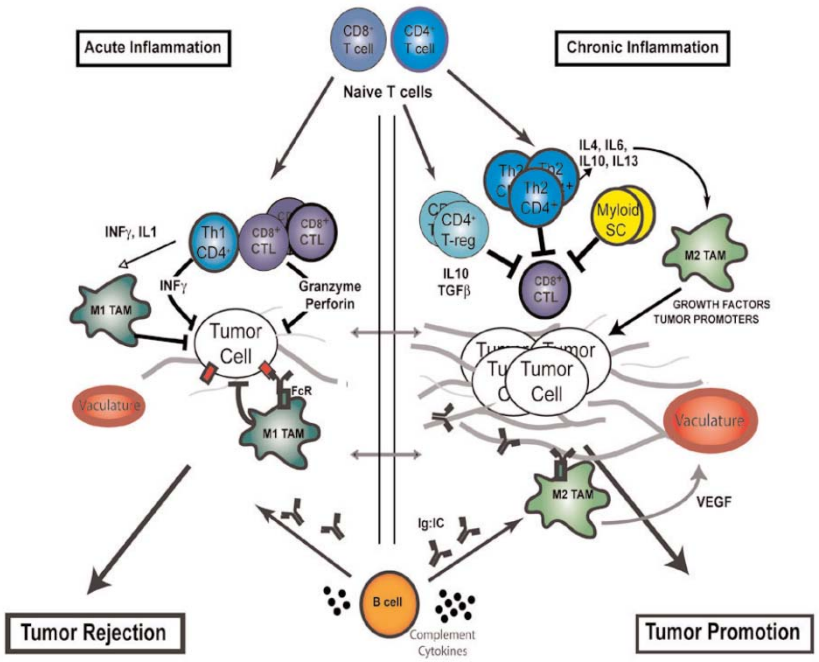

Fig.3. Schematic illustration of the two major inflammatory responses associated with cancer development. The left scenario represents the anti-tumor response, in which Th_1 $CD4^+/CD8^+$ T-cells mediate a cytotoxic response in conjunction with pro-inflammatory macrophages (M1), ultimately eradicating the tumor. The right scenario depicts the situation in most solid tumors, in which the cytotoxic Th_2 $CD4^+/CD8^+$ T-cell response is suppressed by pro-tumorigenic macrophages (M2), myeloid suppressor cells (SCs) and activated B-cells and regulatory T-cells (T_{reg}), ultimately promoting tumor growth. Adapted from DeNardo and Coussens, 2007.

The cytokine profile of the tumor microenvironment additionally alters the phenotypes and functions of many innate immune cells. To this end, constitutive transforming growth factor-β (TGF-β) signaling was recently shown to skew the neutrophil phenotype from pro-inflammatory and anti-tumorigenic (N1) to mediate chronic inflammation and becoming pro-tumorigenic (N2), (Fridlender *et al*. 2009). This alternative activation of neutrophils naturally changes their secretome, and rather than fighting the tumor, the N2 phenotype takes on pro-tumorigenic properties similar to those described for macrophages in the following sections

2.2.1-2.2.3. Moreover, the N2 neutrophils effectively suppress $CD8^+$ T-cell maturation, and thus directly induce immune tolerance (Fridlender *et al*. 2009).

2.2 Tumor associated macrophages

Similar to the “neutrophil switch”, macrophages can alternate between pro-inflammatory (M1) and pro-tumorigenic (M2) phenotypes depending on signaling from the local environment (Ting and Rodrigues, 1980, Gordon, 2003, Mantovani *et al*. 2004, Sica *et al*. 2008b, Fridlender *et al*. 2009, Pollard, 2009). Macrophages differentiate from circulating monocytes upon recruitment to – and infiltration of- epithelial tissues in response to colony stimulating factor-1 (CSF-1) signaling (Lin *et al*. 2001). Upon infiltration, they are further stimulated by the local microenvironment to assume either the M1 or M2 phenotype. The M1 phenotype is generally activated by microbial products (e.g. LPS), TNF-α and interferon-γ (IFN-γ). M1 macrophages are antigen presenting, phagocytozing and produce large amounts of the pro-inflammatory cytokines interleukin-12 (IL-12) and interleukin-23 (IL-23), as well as inducible nitric oxide synthase (iNOS) and reactive oxygen intermediates (ROIs) (Mantovani *et al*. 2008, Sica *et al*. 2008b). iNOS and ROIs are on one hand important for T-cell maturation and on another they generate a hostile microenvironment for “non self” such as intruding micro-organisms and tumor cells (Mantovani *et al*. 2008). However, in response to TGF-β, IL-4 - IL-6, IL-10 and IL13, macrophages polarize into a M2 phenotype (Sinha *et al*. 2005a, Sica *et al*. 2008b, DeNardo *et al*. 2009, Bierie and Moses, 2010). A central event in M2 polarization is activation of NFκB, which leads to increased secretion of pro-tumorigenic cytokines such as TNF-α, IL-6 and IL-10, which help sustain a chronic inflammatory response in both paracrine and autocrine manners (Lu *et al*. 2006, Hagemann *et al*. 2008, Sica *et al*. 2008b, Porta *et al*. 2009,

Pollard, 2009, Biswas and Lewis, 2010, Mancino and Lawrence, 2010). M2 polarized macrophages display poor, or completely lack, antigen presentation, and therefore fail to prime $CD4^+$ T-lymphocytes. Moreover, IL-4 and IL-13 induce macrophage production of arginase, thereby depleting the microenvironment of arginine. Arginine is crucial for the production of iNOS and ROIs and the high expression of arginase therefore inhibits differentiation and maturation of naïve T-cells (Sinha *et al*. 2005b). Interestingly, $CD4^+$ T-cells produce large amounts of IL-4 and therefore represent an efficient paracrine feedback loop in which $CD4^+$ T-cells and M2 macrophages support the pro-tumorigenic phenotype (DeNardo *et al*. 2009). Besides mediating immune surveillance, M2 macrophages, much alike other leucocytes and CAFs, are a major stromal source of pro-tumorigenic cytokines, growth factors, and a variety of proteinases (Hagemann *et al*. 2004, Hagemann *et al*. 2005, Lin *et al*. 2006, Sica *et al*. 2008b, Pollard, 2009, Gocheva *et al*. 2010, Kessenbrock *et al*. 2010). Interestingly, experimental inhibition of the Iκκ/NFκB pathway in M2 macrophages induced an M1 phenotype which correlated with increased tumoricidal activity and overall tumor regression. The change in immune response was in part due to increased recruitment of NK-cells and in part due to abolishment of macrophage mediated immune suppression (Hagemann *et al*. 2008). This study highlights the importance of the macrophage polarization in paracrine signaling and orchestration of immune surveillance. Moreover, it provides a proof of principle of how macrophage polarization can be targeted and altered therapeutically (Hagemann *et al*. 2008).

The paradigm of the “macrophage switch” provides a compelling model for macrophage differentiation, however it solely deals with two extremes of a broad spectrum of mononuclear subtypes represented in the highly complex and heterogeneous tumor tissue. This spectrum includes bone marrow derived cells,

myeloid-derived suppressor cells (MSDC, $Gr1^{+}CD11b^{+}$), $Tie2^{+}$ macrophages and M1/M2 macrophages which display partially distinct and partially overlapping features and functions (Fig. 4), (Mantovani *et al*. 2004, Venneri *et al*. 2007, Yang *et al*. 2008, Mosser and Edwards, 2008, Martilla-Ichihara *et al*. 2009, Pollard, 2009). Due to the well documented heterogeneity of myeloid infiltrates in solid tumors some flexibility is being added to the "M1/M2 macrophage dogma" to generate a classification system in which macrophages are part of a continuum of different phenotypes infiltrating the establishing and growing tumor (Pollard, 2009). In this text I will make use of this flexibility and consequently refer to this group of myeloid derived subtypes with monocyte/macrophage properties as tumor associated macrophages (TAMs).

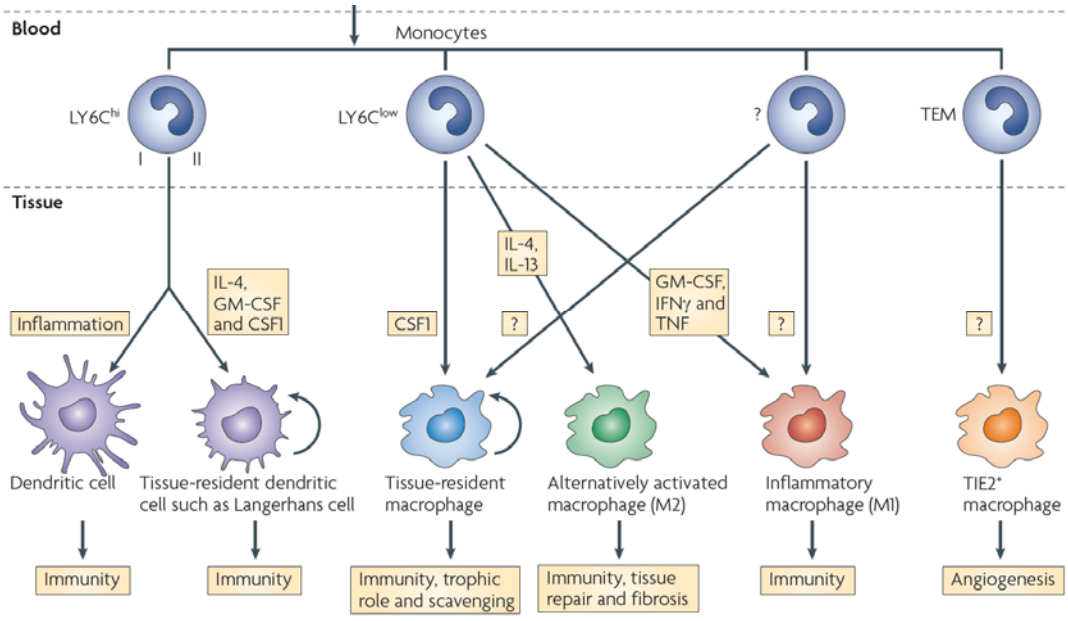

Fig. 4. A schematic illustration of the mononuclear phagocytic cell lineage and the control of macrophage differentiation by growth factors. Circulating monocytes migrate into tissues and differentiate into macrophages of various sub-phenotypes depending on the combination of cytokines present in the local microenvironment. $LY6C^{high}$ monocytes preferentially differentiate into dendritic cells, whereas $LY6C^{low}$ and TEM^{+} monocytes differentiate into macrophages displaying various functions ranging from immunity and tissue repair to neo-angiogenesis. Adapted from Pollard, 2009.

2.2.1 TAMs regulate the angiogenic switch

Tumor growth often exceeds vascular proliferation and as a consequence the tumor develops hypoxic regions. Besides low oxygen tension, hypoxia is associated with low levels of glucose, increased levels of lactate and low pH (Carmeliet and Jain, 2000, Pollard, 2009). These conditions activate the hypoxia inducible factor-1 (HIF-1) pathway resulting in tumor cell differentiation- and proliferation stop and increased secretion of pro-angiogenic factors such as VEGFs (Forsythe *et al*. 1996, Maxwell *et al*. 1997).

VEGFs are endothelial cell mitogens that regulate angiogenesis both through recruitment of endothelial cells and stimulation of their proliferation (Maxwell *et al*. 1997, Du *et al*. 2008). Whereas healthy vessels sprout from established vasculature, which is structurally supported by capillary supporter cells or pericytes, tumor-vessels are characterized by lack of pericyte association, which results in leaky and tortuous vessels (Carmeliet and Jain, 2000, Greenberg *et al*. 2008).

VEGFs are also potent chemoattractants for TAMs (Leek *et al*. 2000). Tissue histology of various tumor types has demonstrated that TAMs are recruited to- and aggregate in hotspots- located within avascular tumor regions (Leek *et al*. 2000, Lewis *et al*. 2000). Although tumor cells can induce neo-angiogenesis by secretion of VEGF, a major contribution of VEGFs originates from TAMs. This has been shown in various murine tumor models where TAM infiltration was abrogated either by silencing of the *Csf-1* gene ($Csf^{op/op}$), or by pharmacologic depletion, with the result of reduced tumor burden and significant decrease in angiogenesis (Lin *et al*. 2006, Zeisberger *et al*. 2006).

Besides VEGF signaling, TAMs contribute to tumor angiogenesis by secreting MMPs and cathepsins involved in ECM remodeling, thereby making way for

sprouting vessels (Hagemann *et al*. 2004, Lin *et al.* 2006, Gocheva *et al*. 2010). Thusm TAMs are considered to be key regulators of the “angiogenic switch” during tumor progression (Lin *et al*. 2006).

2.2.2 Preparing the metastatic niche

Genetic (*Csf1*^{op}/^{op}) or chemical (bisphosphonate liposome mediated) ablation of TAM infiltration in spontaneous and orthotopic murine models have also proven to be sufficient to abrogate metastasis formation. These observations assign a central role for TAMs in the metastatic process (Wyckoff *et al*. 2004, Yang *et al*. 2008, Qian *et al*. 2009).

Metastasis depends on a number of rate-limiting steps of which tumor cell intravasation, extravasation, colonization and outgrowth are the most significant. Invasive tumor cells intravasate into the blood stream mainly through the leaky tumor-vasculature. Once in the circulation system, tumor cells propagate to distant sites where they extravasate into new host tissues to form metastatic colonies (Joyce and Pollard, 2009).

The tissue preference of metastasizing cells is highly cancer type specific (Bos e*t al*. 2009, Nguyen *et al*. 2009, Zhang *et al*. 2009). Gene expression analyses of cells isolated from breast cancer patients with brain and lung metastasis have revealed distinct signatures of primary and metastatic cancer cells, as well as between lung and brain metastases (Bos *et al*. 2009). For example, cyclo-oxogenase 2 (COX-2) and heparin binding EGF was highly expressed in tumor cells extravasating into lung and brain, but not in cells extravasating into bones and lever. Moreover, tumor cells extravasating to the brain showed high expression of the α2.6-sialyltransferase ST6GALNAC5, suggesting that surface sialylation can be determining for whether disseminating breast cancer cells

extravasate into the brain or to e.g. lungs (Bos *et al*. 2009). Moreover, cell type specific activation of pathways signaling survival and proliferation upon extravasation was identified and whereas breast cancer engraftment in bone was shown to depend on activation of the non-receptor tyrosine kinase Src, bone engraftment of lung adenocarcinomas was mediated partially by activation of the Wnt-pathway (Nguyen *et al*. 2009, Zhang *et al*. 2009).

Metastatic gene expression signatures and activation of specific signaling pathways are important for tumor cell survival during circulation and tissue extravasation, however the host environment is crucial for colonization and metastatic outgrowth. Extravasating cells experience oxidative stress and to counteract this they recruit $CD11b^{+}VEGFR^{+}$ TAMs (Kaplan *et al.* 2005, Kaplan *et al*. 2007, Erler *et al*. 2009). Through extensive paracrine signaling, infiltrated TAMs induce angiogenesis, suppress cytotoxic immune responses, and stimulate tumor cell growth and proliferation. The colonization by metastatic tumor cells in foreign tissue therefore largely depends on the establishment of such TAM-mediated metastatic niches. The observation that tumor metastasis is abrogated upon genetic ablation or pharmacologic depletion of TAMs in murine models highlights the importance of myeloid recruitment, paracrine signaling and the metastatic niche in disease progression.

2.2.3 Orchestrating tumor invasion

The metastatic niche theory deals with events occurring during tumor cell circulation, extravasation and colony outgrowth, but not with the initiating event; tumor cell intravasation. The tumor vasculature plays a central role in this process as its leaky nature provides an obvious escape path for invasive tumor cells. The blood stream not only provides an escape path for disseminating tumor cells but

also an entry point for circulating non-malignant stroma cells. The tumor derived cytokines IL-4, RANTES and CCL2 stimulate an autocrine TNF-α loop in infiltrating TAMs resulting in an increased secretion of MMPs and cathepsins. These proteases further contribute to tumor cell intravasation as they through enzymatic digestion perforate the basement membrane and the endothelial barrier (Hagemann *et al*. 2004, Gocheva *et al*. 2006, Gocheva *et al*. 2010).

Local tumor expansion likewise depends on proteolytic digestion of the ECM. Moreover, tumor cells have to migrate to- and proliferate within- the invasive front. This process is orchestrated via reciprocal tumor cell-TAM relay loops of CSF-1/EGF, CSF-1/ SDF-1 and via the CCL5/RANTES axis (Wyckoff *et al*. 2004, Karnoub *et al*. 2007, Green *et al*. 2009, Joyce and Pollard, 2009, Patsialou *et al*. 2009, Qian *et al*. 2009). In this sense, proteolytic degradation of the ECM mines the path, while TAM-derived chemokines lead the way for migratory tumor cells. The paracrine signaling network between tumor cells and TAMs thus provides important extrinsic regulation of tumor invasion and dissemination (Fig. 5).

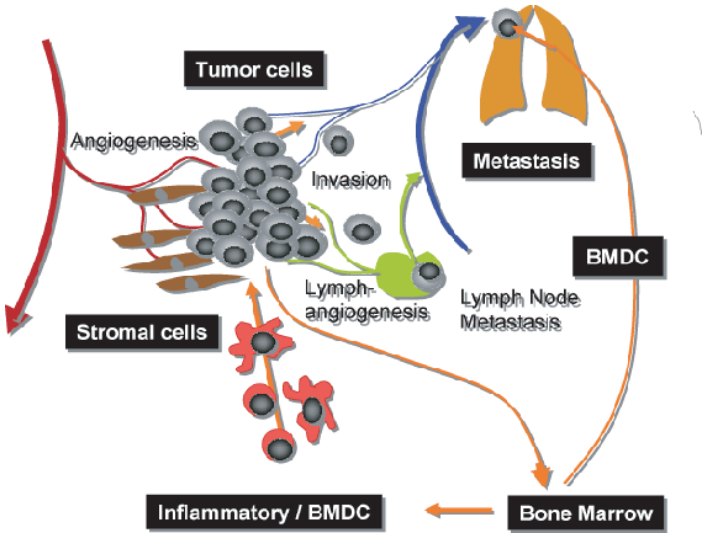

Fig. 5. Schematic illustration of the tumor promoting activities of inflammatory and bone marrow derived cells, including onset of the angiogenic switch (lymph and hem), promotion of tumor cell invasion and preparation of the metastatic niche. Adapted from Rüegg C. 2006.

2.3 Becoming motile; intrinsic regulation of tumor cell invasion

A crucial step in tumor invasion is a phenotypic transition through which adherent epithelial cells acquire migratory properties. Several distinct pathways can mediate such trans-differentiation but common for them is that they induce changes in the expression of adherence molecules, reorganization of the cytoskeleton and formation of proteolytic membrane protrusions required for invasion of the surrounding environment as well as for chemotaxis (Friedl, 2004, Kedrin *et al*. 2007, Yilmatz and Christofori, 2009). Although the dynamics of cell migration have been widely studied in the context of development and pathology, the means of regulation in a tumor context remain elusive. The following sections review the key steps in the different cellular pathways employed in the process of cell migration.

2.3.1 The amoeboid approach; generation of Invadopodia

The term invadopodia generally refers to membrane protrusions generated by transformed cells in contrast to podosomes, which predominantly refers to protrusions formed in migrating leucocytes (Gimona *et al*. 2008, Yilmatz and Christofori, 2009). A key step in invadopodia formation is reorganization of the actin cytoskeleton. This step is regulated by a complex signaling network involving growth factor receptor signaling (e.g. EGF, HGF, TGF-β), integrin β_1 and β_3 signaling and non receptor tyrosine kinase signaling, mainly Src. Activation of these pathways initially leads to destabilization of the cytoskeleton either through partial or complete disruption of the membranous actin complexes (Kedrin *et al*. 2007, Yamaguchi and Condeelis, 2006, Yilmatz and Christofori, 2009). Subsequent activation of e.g. the phosphoinositide-3-kinase (PI3K) and the

phospholipase Cγ (PLC) pathways leads to downstream activation of the small GTPases RhoA, Rac and Cdc42 (Kedrin *et al*. 2007). Rac is shuffled to the protrusion, also termed the leading edge, where it activates the actin related protein 2/3 complex (Arp2/3) to induce actin nucleation (Kedrin *et al*. 2007, Wang *et al*. 2010). The newly cross-linked actin filaments provide mechanical support for the protrusion, which is necessary to withstand the mechanical shear force provided by the invaded ECM. For complete cell movement, actin filaments located in the trailing edge have to retract, a process regulated mainly by RhoA and Cdc42 (Kedrin *et al*. 2007).

During the formation of new protrusions, integrin receptors are shuffled to the surface of the tip. Here they sense chemokine signaling from the local microenvironment and when stimulated accordingly they establish temporary contact to the underlying ECM (Fig. 6), (Friedl, 2004).

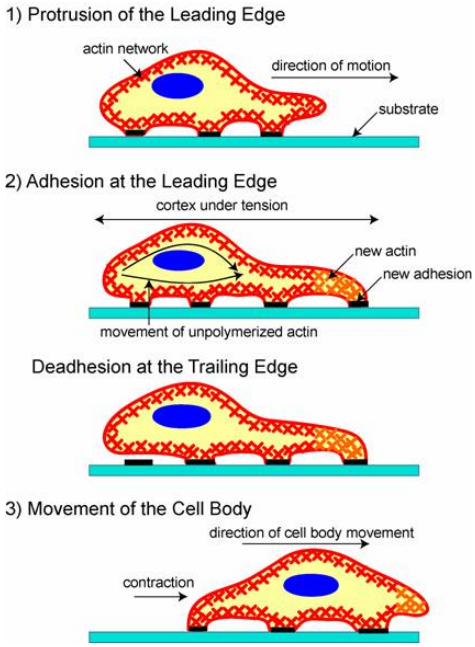

Fig. 6. Schematic illustration of cell migration mediated by protrusions.
1) protrusions are formed in the leading edge and 2) integrins are shuffled to the surface of the tip to mediate adhesion to the ECM while the trailing edge detaches and unpolymerized actin is shuffled to the leading edge. 3) The trailing edge is moved forward due to myosin-mediated contractility, and cell has successfully moved ahead. Adapted from: http://www.biolsci.org/getimage.php

A recent report investigating Rac activation in drosophila border cells have identified an interdependency between localization of Rac to the protrusions and activation of tyrosine kinase receptors, such as EGFR. This finding may suggest that whereas Rac is important for actin nucleation in the forming invadopodia, activation of tyrosine kinase receptors may be important for chemotaxis in a similar manner as integrin β_1 and β_3 (Wang *et al*. 2010).

A distinct feature of the invadopodia is their ability to degrade the ECM. Membrane type MMPs (Mt-MMPs) and serine protease uro-kinase plasminogen activator (uPA) and its receptor (uPAR) are recruited to the leading edge of the cells from where they actively participate in degradation of the surrounding tissue (Friedl, 2004, Yilmatz and Christofori, 2009).

The ultimate inducing event leading to the formation of invadopodia remain elusive, however it is well acknowledged that membrane protrusions can arise spontaneously when tumor cells are grown on an appropriate substrate such as collagens naturally found in the ECM. This could suggest the involvement of a positive feed forward mechanism linking ECM remodeling and activation of integrins to the initial formation of invadopodia (Gimona *et al*. 2008).

2.3.2 Following the lead; cluster invasion

Epithelial cells can either migrate as single cells or in clusters. Cluster invasion is mainly known from embryogenesis and wound healing, but the phenomenon has also been reported for various cancers during tissue invasion and intravasation into the circulation system (Friedl and Gilmour, 2009).

Cluster invasion requires partial cell-cell cohesion, collective cell polarization and intercellular coordination of cytoskeletal remodeling activity. The protrusions

are formed mainly in the leading cells, whereas cells located in the rear end display a predominant retractile phenotype. Cells located between the front and the rear end can adapt completely, partially or not at all, depending on their position. The centre cells thus vary from displaying temporary or partial protrusion formation, localized enzymatic activity or an adherent epithelial phenotype (Fig. 7). Cluster invasion thus provides a functional mechanism for invasion by cells that by nature are immotile or display poor migratory properties (Friedl and Gilmour, 2009).

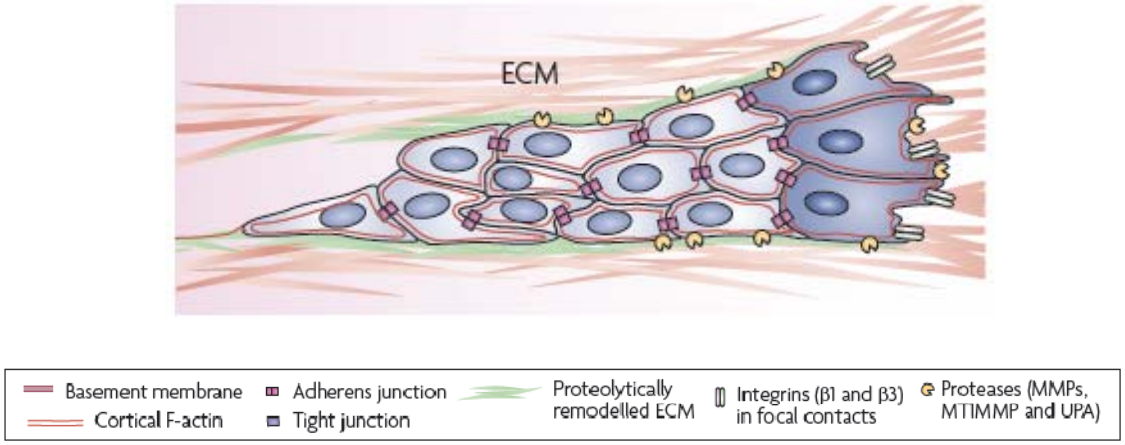

Fig. 7. Schematic illustration of collective invasion in the ECM by detached cancer cells which are moving as a small cluster. The leader cells (blue cells to the right) display, as the only cells in the cluster, taxis sensing and proteolytic protrusions. Other cells in the cluster are directly or indirectly attached to the leader cells through adherence junctions, and only the cells lining the cluster display proteinase activity and partial membrane protrusion. Adapted from Friedl and Gilmour, 2009.

The mechanisms controlling and defining the development of cluster invasion are poorly understood (Friedl and Gilmour, 2009). However, recent studies have provided some useful insights e.g. by. identifying Rac as an important intrinsic mediator of cluster invasion (Wang *et al*. 2010). Rac was shown to be differentially expressed among cells in the cluster, and polarized expression in leading cells was sufficient to promote invasion by the entire cluster. As the clustered cells are tied together by epithelial adherence junction, physical disruption hereof may be a

determining factor. The epithelial adherence protein E-cadherin is a known substrate for MMPs and Cathepsins, suggesting that proteinase mediated disruption of adherence junctions, e.g. as a consequence of tissue remodeling may play a role (Gocheva *et al*. 2006, Orlichenko and Radisky, 2008). Extrinsic processing of E-cadherin, in combination with the differential genomic integrity characteristic for mammalian tumors, may determine which cells in a cluster activate Rac and become invasive, and which passively follow the lead) Friedl and Gilmour, 2009, Gocheva *et al*. 2006, Orlichenko and Radisky, 2008).

2.3.3 Going solo; single cell invasion

The alternative to cluster invasion is single cell invasion. Intra-vital imaging of murine tumors recently revealed that rapid and transient exposure of tumor cells to TGF-β1 is sufficient to induce a switch from clustered to single cell invasion (Giampieri *et al*. 2009). Moreover, using this technique it was demonstrated that clustered invasion solely homes to the lymphatic system, whereas single cells intravasates into both lymphatics and the blood stream, the latter resulting in metastasis (Giampieri *et al*. 2009, Giampieri *et al*. 2010).

Single cells can employ either an amoeboid (Fig. 6) or mesenchymal (see section 2.4 and Fig. 8) strategy upon invasion. The two strategies are distinct although they share some common features like complete loss of epithelial adherence junctions, stress fiber generation and morphological changes (Friedl, 2004, Matise *et al*. 2009). Whereas classical amoeboid migration (via podosomes) is associated mainly with leucocyte trafficking, mesenchymal cell migration is the predominant mean of single cell migration in epithelial cancers (Yilmatz and Christofori, 2009, Matise *et al*. 2009). Interestingly, TGF-β has previously been shown to induce mesenchymal trans-differentiation in epithelial cancer cells

through activation of the RhoA pathway, suggesting that the recently identified implication for TGF-β in single cell invasion may be related to its role in epithelial to mesenchymal transition (Bhowmick *et al*. 2001, Matise *et al*. 2009)

2.4 Epithelial to mesenchymal transition

Epithelial cells can acquire mesenchymal features by undergoing EMT (Thiery, 2002, Nawshad *et al*. 2005, Thiery *et al*. 2009, Yang and Weinberg, 2008). Although initially characterized as a crucial mechanism for embryogenesis, it is gaining acknowledgement for its potential involvement in promoting tumor invasion and metastasis (Thiery *et al*. 2009, Yang and Weinberg, 2008). This is in part based on research conducted in a number of cancer cell lines with the ability to undergo such trans-differentiation *in vitro*, and in part on a number of clinical studies successfully correlating EMT marker expression in tumor samples with poor disease outcome (Bakin *et al.* 2000, Lu *et al.* 2003, Gal *et al*. 2008, Soltermann *et al*. 2008, Thiery *et al*. 2009, Yang and Weinberg, 2008).

In brief, cancer cells can trans-differentiate in response to intrinsic events, e.g. genetic mutations, or extrinsic signaling e.g. from the tumor stroma (see section 2.4.4), (Gazit *et al*. 1999, Bakin *et al*. 2000, Bhowmick *et al*. 2001, Lu *et al*. 2003, Thiery and Sleeman, 2006, Gal *et al*. 2008, Thiery *et al*. 2009). As illustrated in fig. 8, the highly organized structure that characterizes epithelial tissues is lost during EMT (dysplasia), as the cells reorganize their cytoskeleton, change morphology and become fibroblast-like (carcinoma *in situ*). Moreover, the cells up-regulate the production of proteases and become invasive (invasive carcinoma), (Thiery, 2002, Yilmatz and Christofori, 2009). Via extensive proteolysis, the trans-differentiated cancer cells break through the basement membrane and intravasate into the circulation system where they travel up against chemokine gradients provided by

pre-metastatic and metastatic niches. Upon arrival to a new host tissue, the cancer cells extravasate into the metastatic niche where they potentially undergo the reverse mechanisms, mesenchymal to epithelial transition (MET). MET represents a mechanism through which trans-differentiated cancer cells can regain their epithelial phenotype, colonize and establish micro metastasis (Fig. 8), (Thiery, 2002, Hugo *et al*. 2007, Thiery *et al*. 2009).

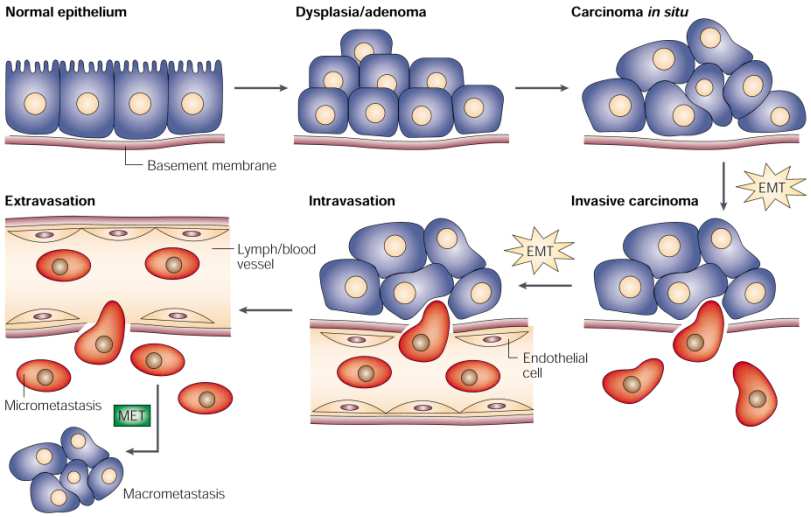

Fig. 8. Schematic illustration of the cellular changes of epithelial tumor cells undergoing EMT. At the state of complete trans-differentiation, the cells are highly invasive and can intravasate into the circulation system. Upon extravasation the cells undergo MET to regain epithelial traits. Adapted from Thiery, 2002.

EMT may also play a crucial role during local tumor invasion. Instead of intravasation, the trans-differentiated cells may use their proteolytic activity to degrade the ECM. Chemotaxis allows them to migrate towards the invasive front of the tumor, constantly being on the edge to the surrounding tissue (Thiery, 2002, Thiery *et al*. 2009).

Besides tumor cell invasion, EMT is thought to confer a drug resistance potential as well as stem cell like properties (Mani *et al*. 2008, Thiery *et al*. 2009, Polyak and Weinberg 2009, Santisteban *et al*. 2009, Reiman *et al*. 2010). Moreover, EMT has been linked to increased production of reactive oxygen

species (ROS), consequently facilitating oxidative DNA damage and genomic instability (Radisky *et al*. 2005, Inumaru *et al*. 2009, Zhou *et al*. 2009). It has therefore been speculated that abrogation of tumor cell EMT could be of value for successful future therapies (Mani *et al*. 2008, Polyak and Weinberg, 2009, Thiery *et al*. 2009).

2.4.1 Molecular hallmarks of EMT

A key step in EMT is loss of E-cadherin. Whereas the extracellular domain of this transmembrane glycoprotein mediates homophillic adherence junctions with neighboring cells, the intracellular domain forms complexes with the transcriptional co-activator β-catenin and the cytoskeleton linker protein α-catenin (Nawshad *et al*. 2005). Upon loss of E-cadherin the complex dissociates, the proteins are released, the cytoskeleton rearranges and β-catenin can associate with transcription factors of the T-cell factor/lymphoid enhancer factor (Tcf/Lef) families and induce transcription of, among other, mesenchymal hallmark genes such as N-cadherin, the intermediate filament vimentin and the ECM component fibronectin (Korinek *et al*.1997, van de Wettering *et al*. 2002, Schmidt-Ott *et al*. 2007). E-cadherin can be transcriptionally repressed by members of the SNAI (e.g. snail and slug), bHLH (e.g. twist) and ZEB (zeb1/2) families, which meditate suppression by directly binding to the E-box sequence in the E-cadherin promoter (Cano *et al*. 2000, Chua *et al*. 2007, Vesuna *et al*. 2008).

Loss of E-cadherin often correlates with expression of N-cadherin, a phenomenon referred to as the “ cadherin switch” which, along with up-regulation of snail and twist, is regarded molecular signatures of EMT (Maeda *et al*. 2004, Nawshad *et al*. 2005).

2.4.2 Signaling EMT

EMT can be regulated through a number of intrinsic signaling pathways, including but not limited to tyrosine kinase receptor signaling, e.g. EGFR, Wnt/β-catenin, Notch, integrin mediated and TGF-β receptor signaling (Gazit *et al*. 1999, Bakin *et al*. 2000, Bhowmick *et al*. 2001, Lu *et al*. 2003, Thiery and Sleeman, 2006, Gal *et al*. 2008, Thiery *et al*. 2009). The signaling cascades required to induce EMT are highly context dependent and although activation of one pathway can be sufficient to incite trans-differentiation, multiple pathways are often found to signal in conjunction to mediate a sustainable response (Nawshad *et al*. 2005, Thiery and Sleeman, 2006, Lo *et al*. 2007, Dissanayake *et al*. 2007, Labbé *et al*. 2007, Uttamsingh *et al*. 2008, Vincent *et al*. 2009).

In a simplified view, the many EMT regulating pathways converges at the transcriptional level. Essentially, activation of the surface receptors results in up-regulation of snail and/or twist, which mediate suppression of E-cadherin. As a consequence the adherence complexes dissociates and the transcription factors NFκB and β-catenin, which are normally sequestered in the complex, are released (Solonas *et al*. 2008) . Moreover, EMT signaling can inhibit protein degradation by mediating phosphorylation, and thus inactivation, of glycogen synthase kinase-3-β (GSK3β). Consequently NFκB and β-catenin are stabilized and can translocate to the nucleus to initiate the EMT transcription program (Fig. 9), (Thiery and Sleeman, 2006).

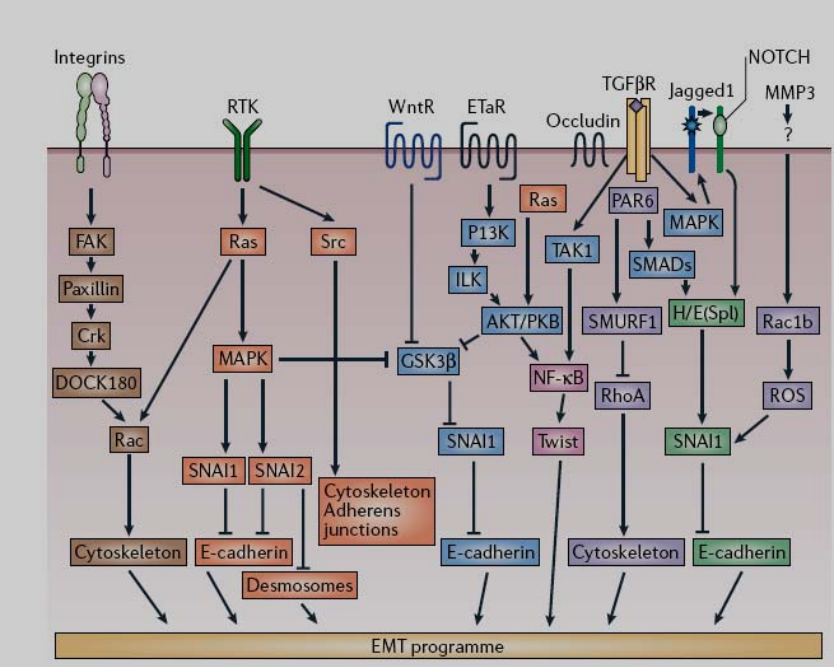

Fig. 9. Schematic illustration of selected molecular networks and their crosstalk, involved in regulation of EMT. Receptor tyrosine kinases (RTKs) are known to induce EMT in several epithelial cancer cell lines, however its signaling highly depends on co-activation of integrin receptors. Together they activate Rac, which induces cytoskeleton remodeling as described in section 2.3.1. RTKs furthermore signal EMT through sequential activation of MAPK and snail mediated repression of E-cadherin and consequential induces onset of the EMT transcription program. Canonical Wnt signaling mediates stabilization of snail through direct inhibition of GSK3β. Snail represses E-cadherin, which in turn releases β-catenin and NFκB. Upon translocation to the nucleus they initiate the EMT transcription program. TGF-β signaling has been extensively studied for its involvement in EMT *in vitro* and *in vivo*. The canonical pathway signals through SMAD, which induces snail mediated E-cadherin repression. The non-canonical signaling path for TGF-β goes through PI3K/Akt and RhoA. PI3K/Akt activation induces a NFκB response and twist mediated E-cadherin repression, whereas signaling through RhoA mainly induces cytoskeleton remodeling as described in section 2.3.1. Notch signaling has gained recent acknowledgement for its potential to induce EMT, however the pathways through which it regulates EMT are still under investigation. MMP3 induces EMT through activation of Rac1b, which further induces the production of ROS consequentially stabilizing snail and repressing E-cadherin. Adapted from Thiery and Sleeman, 2006.

EMT can also be induced as a consequence of inherited or acquired genetic mutations. Naturally, mutations in the cytokine receptors can activate EMT signaling, but also downstream mutations can result in constitutive activation of the EMT transcription program. An example hereof is colon cancer, which predominantly is caused by loss-of-function mutations in the tumor suppressor adenomatosis polyposis coli (APC) gene or gain-of-function mutations in β-catenin (Korinek *et al*. 1997, Morin *et al*. 1997, Brabletz *et al*. 2001). Moreover, a recent study identified a correlation between common K-Ras mutations and EMT

signaling pathways, suggesting that K-Ras mutations may enhance EMT inducing signaling (Singh *et al*. 2009). The importances of such synergies are further supported by the observation that Ras-transformed hepatocytes, kidney (MDCK) and mammary epithelial (EpH4) cells, to mention a few, only undergo complete EMT upon co-activation of the TGF-β pathway (Oft *et al*. 1996, Janda *et al*. 2002, Tse and Kaluri, 2007).

2.4.3 TGF-β; Dr. Jekyll and Mr. Hyde

TGF-β was originally identified as a tumor suppressor gene and it is a potent controller of cell proliferation and apoptosis (Massague, 1998). The canonical signaling pathway involves SMAD activation, which upon nuclear translocation induces transcription of targets genes. However, for example as a consequence of Ras-mutations or tyrosine kinase receptor activation, TGF-β can switch to signal through non-canonical pathways, such as the PI3K/AKT and/or the RhoA pathways and rather than suppressing tumor growth, TGF-β induces single cell invasion (Fig. 9), (Bakin *et al*. 2000, Bhowmick *et al*. 2001, Akhurst and Derynck, 2001, Nawshad *et al*. 2005, Thiery and Sleeman, 2006, Gal *et al*. 2008, Giampieri *et al*. 2009, Matise *et al*. 2009).

TGF-β has been extensively investigated for its role in EMT and on this note, it was not surprising that TGF-β was identified as a key factor inducing single cell migration instead of clustered migration (Giampieri *et al*. 2009, Giampieri *et al*. 2010). However, the notion that TGF-β induced single cell migration ultimately leads to metastasis at distant sites may highlight the detrimental consequences of tumor cell EMT. Although the significance of EMT in tumor cell invasion is generally accepted, it is important to note that it mainly is based on *in vitro* studies, and that the *in vivo* evidence is limited mainly to histo-pathologic correlation

studies of tumor samples from patients (Bakin *et al*. 2000, Bhowmick *et al*. 2001, Soltermann *et al*. 2008, Thiery *et al*. 2009, Vincent *et al*. 2009). This is in part due to the transient nature of EMT, which makes it difficult to detect trans-differentiated cells with existing visualization techniques. But it is also due to the fact that epithelial cells that have undergone complete EMT, morphologically and phenotypically resemble myofibroblasts, which makes it difficult to distinguish between the fibroblast-like cells of mesenchymal and epithelial origin (see section 2.1.1), (Franci *et al*. 2006, Radisky *et al*. 2007, Kalluri and Zeisberg, 2006, Tse and Kalluri, 2007). As a consequence we know a lot about the intrinsic regulation of tumor cell EMT, but in reality we know very little about its extrinsic regulation in a tumor context *in vivo*.

2.4.4 Stromal regulation of EMT

Histo-pathology analyses of human tumor specimens have revealed a rather large population of mesenchymal-like tumor cells in the invasive front where they closely associate with components of the tumor stroma. This observation has been interpreted as a sign of extensive tumor-stroma communication inciting the phenotypic conversion of epithelial tumor cells to invasive mesenchymal cells (Brabletz *et al*. 2001, Franci *et al*. 2006, Sheehan *et al*. 2008). The tumor microenvironment is a constant source of growth factors and cytokines such as TGF-β, EGF and Wnt to mention a few, that potentially can signal EMT through canonical receptor activation (Gazit *et al*. 1999, Bakin *et al*. 2000, Bhowmick *et al*. 2001, Lu *et al*. 2003, Gal *et al*. 2008, Vincent *et al*. 2009). To this end, it has been suggested that Th_2 polarized $CD4^+/CD8^+$ T-lymphocytes, possibly through paracrine TGF-β signaling, can contribute to an EMT inducing tumor microenvironment and enhance invasion *in vivo* as well as promote disease

recurrence (Santisteban *et al*. 2009, Reiman *et al*. 2010). Disease relapse is generally associated with cancer stem cells, and to this end several reports have identified extensive similarities between trans-differentiated epithelial cells and cancer stem cells (Mani et al. 2008, Polyak and Weinberg, 2009, Santisteban *et al*. 2009, Reiman *et al*. 2010). Depletion of $CD8^+$ T-cells in a murine mammary tumor model completely abolished EMT and prevented relapse, underscoring the importance of $CD8^+$ T-cells both in the regulation of tumor EMT and stem-like cell induced disease recurrence. However, the mechanisms of action are poorly understood, and although Th_2 polarized T-cells is a strong source of TGF-β, its involvement in T-cell regulated EMT remains speculative (Reiman *et al*. 2010).

Stroma-derived proteinases, particularly MMP3, MMP9 and Cathepsin B, can cleave E-cadherin and induce its internalization via endosomes, which alone can be sufficient to induce an EMT response (Fig. 9), (Radisky *et al*. 2005, Gocheva *et al*. 2006, Orlichenko and Radisky, 2008, Gocheva *et al*. 2010). Moreover, MMP-3 has been shown to regulate alternative splicing of Rac1, generating Rac1b, which in turn stimulates the production of ROS and induce oxidative DNA damage, thereby facilitating genomic instability. This pioneer study was the first to link the molecular pathways of EMT to genomic instability (Radisky *et al*. 2005). Recent studies have further confirmed this link, however it has become evident that whereas ROS production is sufficient to induce a temporary EMT response characterized by internalization of adherence molecules from the plasma membrane, it is not enough to induce a sustainable EMT phenotype (Inumaru *et al*. 2009, Zhou *et al*. 2009). Interestingly, the sustainability of ROS induced EMT seems to depend on additional stimuli from e.g. TGF-β and constitutive activation of the pro-inflammatory NFκB-pathway (Tobar *et al*. 2010). Collectively, these reports give way for the possibility that tumor cell EMT may be regulated by tumor

inflammation, a possibility that needs further investigation but hold interesting and promising potential in terms of developing novel adjuvant therapies. The studies emphasize the exiting need to further explore this complex tumor-stroma crosstalk to identify the stromal sources of factors regulating EMT associated tumor progression.

3 Aim(s) of study

As reviewed above, the tumor microenvironment plays crucial and versatile roles in tumor progression. Therapeutic targeting of the tumor stroma therefore holds great promises for improved cancer management. However, the success of such therapies highly depends on careful identification and characterization of the multiple players and molecular processes involved in the various steps of tumor progression.

Our laboratory has previously developed a technique utilizing bisphosphonate-liposomes to deplete TAMs in tumor bearing mice. This technique has been used with great success to investigate and characterize the many and versatile implications for TAMs in tumor promotion (Zeisberger *et al*. 2006, Yang *et al*. 2008, Qian *et al*. 2009). Moreover, in conjunction with molecular as well as cell biological techniques, it has formed the basis for my PhD project, in which I have sought to identify novel implications for TAMs in tumor progression.

Essentially the aims of this study can be condensed into three main points, where aim two and three are direct consequences of aim one, namely:

1) To identify novel implications for TAMs in tumor progression
2) To investigate the regulatory role of TAMs in tumor cell EMT
3) To validate the clinical relevance of TAM regulated tumor EMT

4 Results

The data presented in this section represents my PhD work (Dec. 2006 - Aug. 2010). The experiments were mainly conducted at the Institute of Molecular Cancer Research, University of Zürich. The micro array experiments were performed in collaboration with the Functional Genomics Center Zürich and the analysis of the clinical data were done in collaboration with two pathologists, MD. Alex Soltermann and MD. Verena Tischler, Department of Surgical Pathology, University Hospital Zürich. All institutions are situated in Zürich, Switzerland. The supplementary material is enclosed in Appendix 1, the quality assessments of the mRNA used for the micro array experiment is shown in Appendix 2, and the title page of the manuscript and the abstract is enclosed as Appendix 3.

4.1

Macrophage depletion; proof of principle

To identify novel roles for TAMs in tumor progression, gene expression patterns in F9-teratocarinoma tumors infiltrated by- or depleted of- macrophages were analyzed. The F9-teratocarcinoma model represents a good model for this study as the F9-cells are plastic cells that are sensitive to stimuli by certain cytokines *in vitro* (Krawetz and Kelly, 2008). Moreover, subcutaneously implanted F9-tumors are heavily infiltrated by TAMs and the model is thus susceptible to pharmacologic TAM depletion (Zeisberger *et al*. 2006).

Macrophages were depleted in tumor bearing mice using a liposome based technique, previously established by our group (Zeisberger *et al*. 2006). In brief, the bisphosphonate compound clodronate is intracellularly incorporated into non-hydrolysable analogues of adenosine triphosphates (ATPs), which ultimately leads the affected cells into apoptosis (Selander *et al*. 1996), (Fig. 10A). Clodronate has,

due to its negative charge, a high affinity to bone matrix, and it mainly accumulates in the bones when administered in its free form *in vivo* (Graham and Russell, 2007). Accumulation in the bone matrix can be prevented by encapsulating clodronate in liposomes (clodrolip). Encapsulation furthermore increases the circulation time and allows the drug to be deposited in tissues and organs other than bone. Most importantly, liposomes are phagocytozed by macrophages, and thus the technique provide a tool for macrophage depletion *in vivo* (Zeisberger *et al*. 2006)

The cytotoxicity of clodrolip and empty control liposomes was assessed in a live cell viability assay using M2 polarized RAW 264.7 macrophages *in vitro* (Fig. 10B), (Wang *et al*. 2007). Having confirmed the cytotoxic effects of clodrolip, and lack of toxicity of the empty control liposomes *in vitro*, the liposomes were applied in tumor bearing mice *in vivo*. F9-teratocarcinoma bearing SV129J1 mice were treated with empty liposomes (control) or with clodrolip every 3rd day (1 mg/20g body weight) for three weeks, isolated and subjected to preliminary micro array analyses (Fig. 10C). During the course of treatment the development of the tumors were followed by mean of size estimation (data not shown). At the end of the experiment, the mean tumor volume was significantly smaller in clodrolip treated tumors relative to control tumors (Fig. 10D).

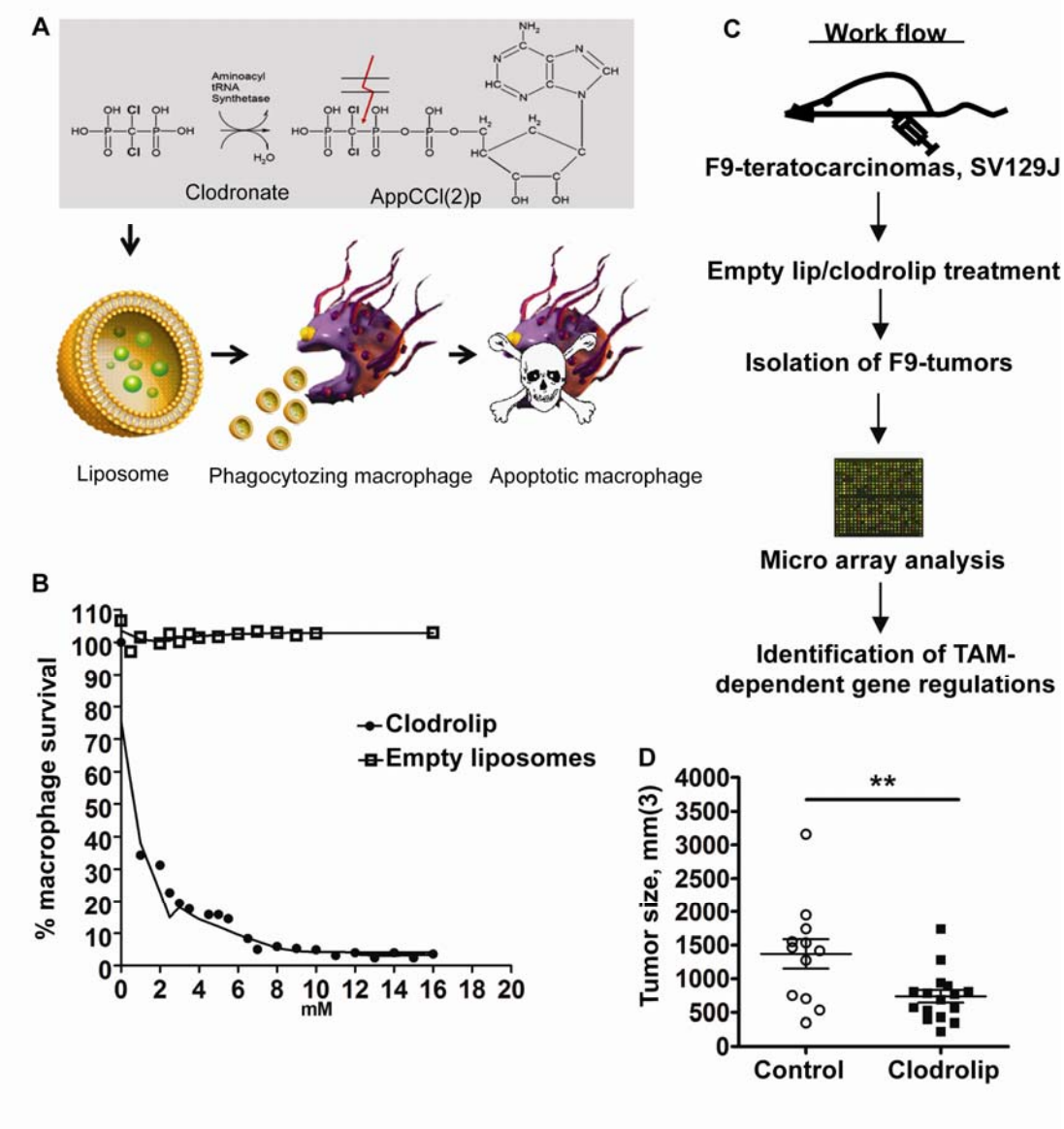

Fig. 10. Assay design and generation of control and macrophage depleted F9-tumors. A. Schematic illustration of the basic principle of clodrolip mediated macrophage depletion. Clodronate is incorporated into non-hydrolysable ATPs. By encapsulating clodronate in liposomes, which are mainly taken up by phagocytozing cells, clodronate (clodrolip) is efficiently delivered to the macrophages. Upon phagocytosis of clodrolip, macrophages undergo apoptosis. **B.** Live cell viability assay (24h) estimating the cytotoxicity of clodrolip and empty liposomes on M2 polarized RAW264.7 macrophages *in vitro*. **C.** Schematic illustration of the work flow implemented to identify novel roles for TAMs in tumor progression. F9-teratocarcinoma tumors were implanted on the flanks of SV1291J mice that were treated over the course of 3 weeks with either clodrolip or empty liposomes. After termination, the tumors were isolated and subjected to micro array analysis. **D.** Tumor size (mm3) distribution at the day of termination of the experiment, in control mice or clodrolip treated mice. ** represents $p < 0.005$, unpaired *t*-test.

Clodrolip treated tumors were significantly smaller than control tumors by the end of the study (Fig. 10D). To assess if the reduction in tumor growth correlated with depletion of TAMs, a comparative gene expression analysis of monocyte/macrophage specific markers was performed. The microarray data revealed a marked decrease in expression of the macrophage specific markers CSFR-1 (~ 5,5 fold), CD68 antigen (~ 3 fold), mannose receptor c (~ 9 fold) and monocyte to macrophage differentiation factor (~ 2 fold) in clodrolip treated tumors relative to controls (Fig. 11A and Table 1). Quantitative real-time PCR (q-PCR) analysis confirmed a reduction in expression of CSFR-1 (~ 4 fold) and CD68 (~ 2 fold) in clodrolip treated tumors relative to controls (Fig. 11B).

Finally, macrophage depletion was confirmed by immunohistochemical analysis of the macrophage specific markers F4/80 and CD68 (Fig. 11C). Our group has previously demonstrated that TAM depletion reduces tumor growth as a consequence of reduced tumor-angiogenesis (Zeisberger *et al*. 2006). To this end, the micro array analysis confirmed a correlation between expression of macrophage specific markers and expression of genes involved in angiogenesis. Whereas the endothelial cell specific receptor, endothelin receptor B and thrombospondin 1 were down regulated in clodrolip treated tumors (~ 3 fold, and ~ 5 fold, respectively), the anti-angiogenic procollagen 18 was up-regulated relative to control tumors (~ 2 fold), (Fig. 11D). Clodrolip-mediated reduction of angiogenesis was further confirmed by immunohistochemistry, analyzing the expression of the endothelial cell specific antigen, CD31 (data not shown). Thus, we concluded that clodrolip efficiently depleted TAMs in the F9-tumors and that depletion was sufficient to abrogate biological TAM regulated processes, such as angiogenesis, important for tumor growth.

Table 1: Relative expression of selected genes in F9-tumors in response to clodrolip mediated TAM depletion.

Category	Gene Bank ID	Gene name	Protein	Fold Change, Clodrolip/Control	Biology
Macrophage markers	AI323359	*Csf1r*	Colony-stimulating factor 1 receptor	-5.5	Macrophage differentiation and function
	BC021637	*Cd68*	CD68 antigen	-3.2	Macrophage expressed glycoprotein
	NM_008625	*Mrc*	Mannose Receptor C type 1	-9.35	Immune receptor facilitating endocytosis of glycoproteins by macrophages
	BC021914	*Mmd*	Monocyte to mac. Dif.	-2.1	Macrophage expressed glycoprotein
Angiogenesis	BF100813	*Erdnrb*	Endothelin receptor B	-2.95	Stimulates endothelial cell migration and proliferation
	AI385532	*Thbs1*	Thrombospondin 1	-4.88	
	D17546	*Col18a1*	Procollagen 18	2.2	Anti-angiogenic
ECM remodeling	NM_008608	*Mmp14*	Matrix metallopeptidase 14	-2.4	Proteolytic degradation of the ECM
	M14222	*Ctsb*	Cathepsin B	-2.4	Proteolytic degradation of the ECM
	NM_007801	*Ctsh*	Cathepsin H	-4.5	Proteolytic degradation of the ECM
	BG064539	*Fn*	Fibronectin	-3.26	Extracellular matrix protein
	BG970109	*Lamb1*	Laminin B	-2.1	Basal Laminae component
Cell-cell adherence & Migration	NM_009864	*Cdh1*	E-cadherin	2.6	Epithelial cell glycoprotein, forms cellular adherence junctions
	AV147875	*Vim*	Vimentin	-2.2	Intermediate filament
	NM_019390	*Lmna*	Lamin A	-4.85	Intermediate filament
	AW121933	*CD44*	CD44	-6.77	Hyaluron receptor, interacts with ECM components & enhance cell invasion
	BC010581	*Stmn1*	Stathmin 1	3.9	Microtubule regulating protein, Oncoprotein 18 (*loss of function*).
	NM_026369	*Arpc5*	Actin related protein 2/3 complex, subunit 5	-2.48	Regulation of actin polymerization. Directs cell migration

N=2/group
Gene arrays; Affymetrix

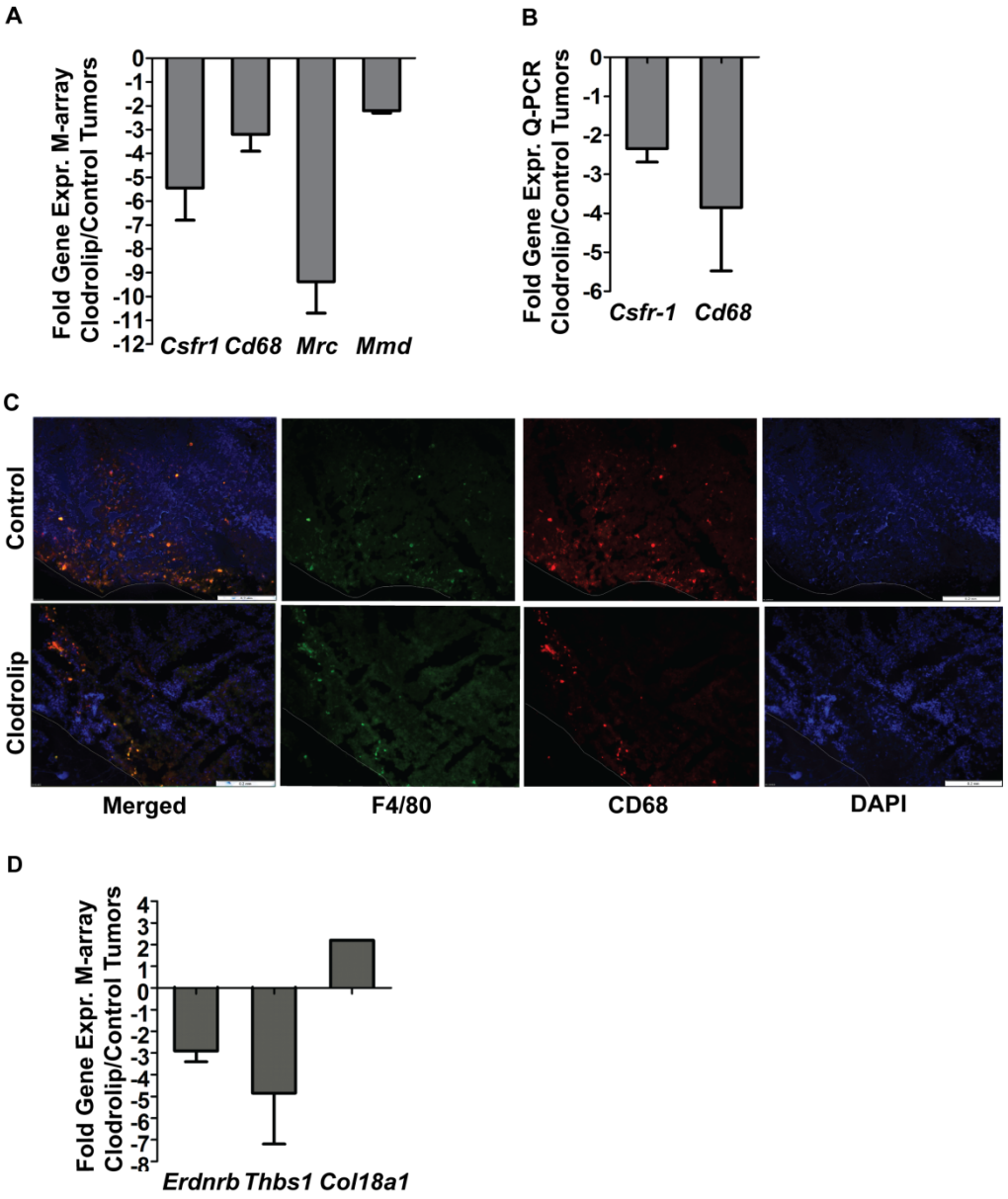

Fig. 11. Clodrolip mediated macrophage depletion in F9-teratocarcinomas. A. Relative fold gene expression (mRNA levels, micro array analysis), of the monocyte/macrophage specific markers CSFR-1 (*Csfr-1*), CD68 (*Cd68*), mannose receptor C (*Mrc*) and monocyte-macrophage differentiation marker (*Mmd*) in clodrolip treated vs. control F9-tumors; n=2, bars:±SEM. **B.** Relative fold gene expression (mRNA levels, q-PCR) of CSFR-1 and CD68 in clodrolip treated vs. control F9-tumors; n=6, bars:±SEM. **C.** Immunohistochemical analysis of F4/80+ (green) and CD68+ (red) density in control (upper panel) and clodrolip treated (lower panel) F9-tumors. Overlay shown in orange; scale bar=0.2 mm. Nuclei were stained with DAPI (blue). **D.** Relative fold gene expression (mRNA levels, micro array analysis), of the angiogenic markers endothelin receptor b (*Erdnrb*), thrombospondin 1 (*Thbd1*) and procollagen 18 (*Col18a1*) in clodrolip treated vs. control F9-tumors; n=2, bars:±SEM.

4.2

Identification of novel roles for TAMs in tumor progression; A link between TAM infiltration and epithelial cell migration

Another important aspect of tumor growth is remodeling of the ECM and to this end, the gene expression analysis revealed a direct correlation between TAM depletion and reduced expression of selected proteases important for proteolytic degradation of the ECM (Fig. 12A and Table 1), (Kessenbrock *et al*. 2010). Besides reduced expression of matrix metalloproteinase 14 (~ 2 fold), cathepsin B and cathepsin H (~ 2 fold, and ~ 4.5 fold, respectively), the matrix protein fibronectin and the basal laminae component, laminin B were reduced ~ 2.5 fold and ~ 2 fold, respectively, in clodrolip treated tumors relative to controls (Fig. 12A). As TAMs are known to be a major source of MMPs and cathepsins in the tumor microenvironment, the relative reduction in expression of proteases in clodrolip treated tumors partially confirmed TAM depletion (Lin *et al*. 2006, Gocheva *et al*. 2010).

Fibronectin can be expressed by mesenchymal stem cells, macrophages and mesenchymal tumor cells (Alitalo *et al*. 1980, Lerat *et al*. 1993, Nawshad *et al*. 2005, Kalluri and Zeisberg, 2006). Its expression in tumor cells is often associated with loss of epithelial adherence junctions, mesenchymal trans-differentiation and acquisition migratory properties (Maeda *et al*. 2004). Indeed, the gene expression analysis revealed an inverse correlation between expression of the epithelial adherence molecule E-cadherin and fibronectin (Fig. 12B). Moreover, a number of genes involved in epithelial cell migration showed altered expression upon clodrolip treatment. The intermediary filaments vimentin and lamin A were both down regulated relative to control tumors (~ 2 fold, and ~ 5 fold, respectively).

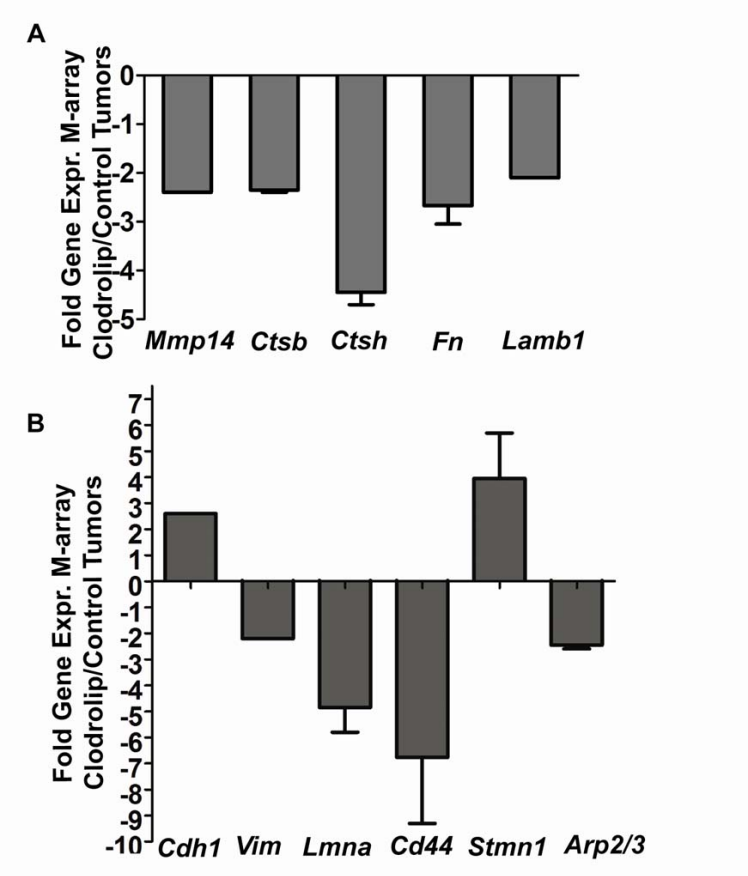

Fig. 12. Clodrolip mediated TAM depletion correlates with gene expression patterns characteristic of epithelial cell migration. A. Relative fold gene expression (mRNA levels, micro array analysis) of factors involved in ECM remodeling in clodrolip treated vs. control F9-tumors; matrix metalloproteinase 14, (*Mmp14*), cathepsin b (*Ctsb*), cathepsin h (*Ctsh*), fibronectin (*Fn*) and laminin b (*Lamb*); n=2, bars = ± SEM. **B.** Relative fold gene expression (mRNA levels, micro array analysis) of factors involved in epithelial cell migration in clodrolip treated vs. control F9-tumors; E-cadherin (*Cdh1*), Vimentin (*Vim*), Lamin A (*Lmna*), CD44 (*Cd44*), Stathmin (*Stmn1*) and actin related protein 2/3 complex subunit 5 (*Arp2/3*); n=2, bars = ± SEM

Expression of the hyaluron receptor CD44, which is important for tumor cell migration, and the actin related protein 2/3 complex, regulating actin polymerization during migration, were similarly reduced ~7 fold and ~2.5 fold, respectively, upon clodrolip treatment (Fig. 12B), (Merzak *et al*. 1994, Mullins *et al*. 1998, Kedrin *et al*. 2007, Outhit *et al*. 2007). In contrast, the migration inhibitor Stathmin 1 was expressed ~ 4 fold higher in clodrolip treated tumors relative to control tumors (Baldasarre *et al*. 2005). All together, the gene expression analysis uncovered a correlation between TAM infiltration and transcriptional regulation of genes involved in epithelial cell migration. In particular, the inverse correlation between E-cadherin and mesenchymal gene expression suggested a potential connection between TAM infiltration and tumor cell EMT.

4.3

Tumor associated macrophages regulate epithelial to mesenchymal transition in tumor cells in a TGF-β dependent manner

The following section is adapted from a manuscript with the same title recently submitted to Clinical Cancer Research (Title page and abstract is enclosed in Appendix 3).

4.3.1

Depletion of TAMs reduces mesenchymal gene expression in murine F9-teratocarcinomas *in vivo*

To address the potential role of TAMs in tumor EMT, clodrolip treated and control F9-tumors were evaluated by q-PCR for gene expression of key markers for EMT (Fig. 13A). The analysis confirmed the micro array data, in that vimentin expression was reduced (~ 5 fold), whereas E-cadherin expression was increased

(~2 fold) in clodrolip treated tumors relative to controls. In addition, the mesenchymal cell adherence molecule N-cadherin was reduced ~ 2 fold in clodrolip treated tumors relative to controls (Maeda *et al*. 2004). Expression of the EMT associated transcription regulators Snail and Twist, both of which repress E-cadherin transcription, were decreased ~2 fold and ~4 fold, respectively, in clodrolip treated tumors (Cano *et al*. 2000, Vesuna *et al*. 2008). Western blotting confirmed a relatively high expression of E-cadherin and its interaction partner β-catenin in clodrolip treated tumors relative to controls (Fig. 13B). To test if the reduction in total β-catenin levels in control tumors correlated with an increase in the active form of β-catenin, an antibody specifically detecting the free, non-phosphorylated form (active β-catenin) was used. However, quantification of the western blot failed to establish obvious differences in the levels of active β-catenin between controls and clodrolip treated tumors (Fig. 13C). Immunohistochemical analysis of the F9-tumors elucidated a correlation between tumor cell expression of selected EMT markers and $CD68^+$ macrophage density (Fig. 13D). Whereas E-cadherin and β-catenin localized to the cellular membrane of tumor cells in areas with low $CD68^+$ densities, both proteins were partially lost in areas with high $CD68^+$ densities. Conversely, fibronectin expression was increased in $CD68^+$ rich areas. TAM density thus correlated with a mesenchymal-like tumor cell phenotype in F9-teratocarcinoma tumors.

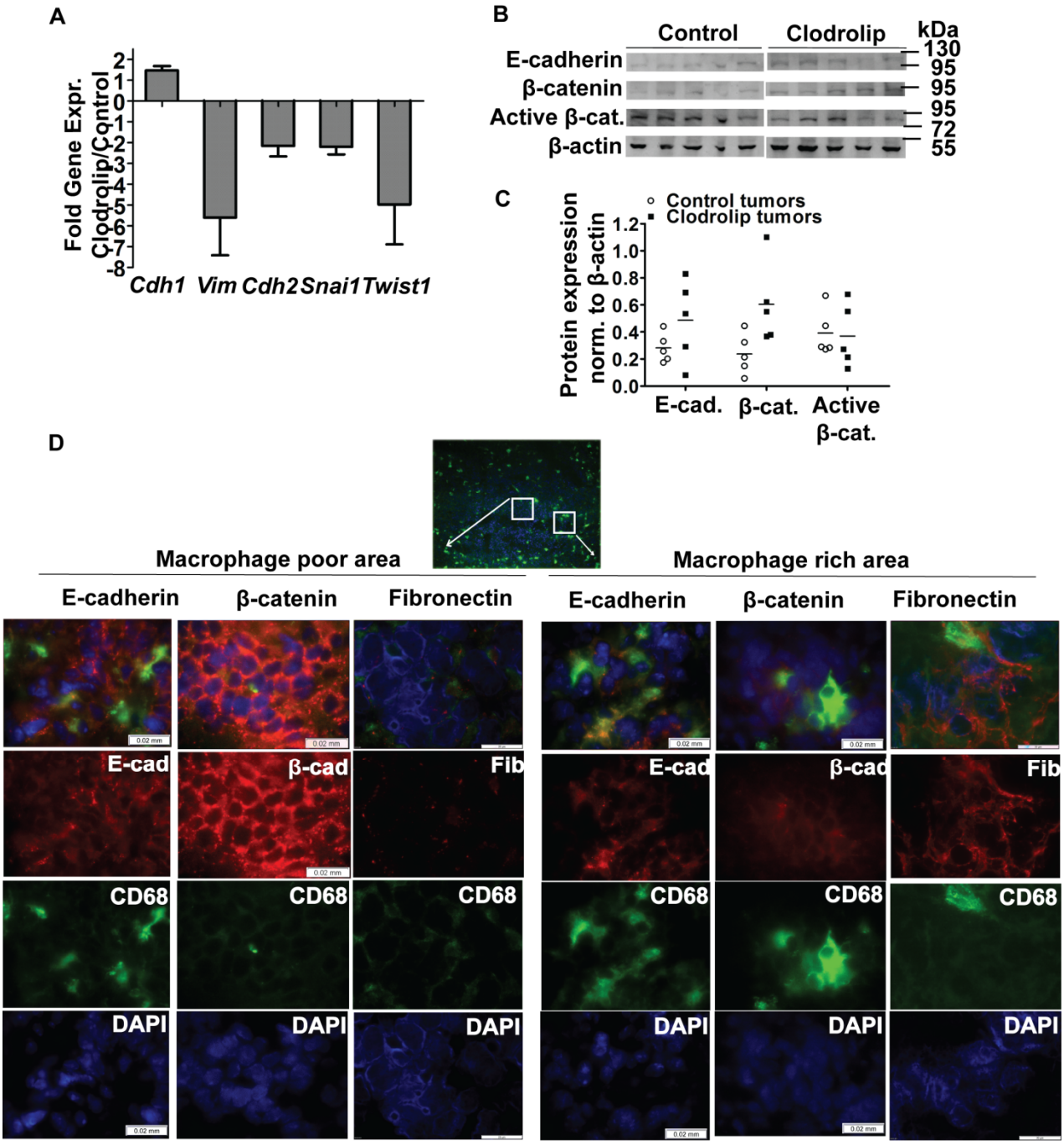

Fig. 13. Depletion of TAMs reduces mesenchymal gene expression in F9-teratocarcinomas. **A**. Fold gene expression (mRNA levels, q-PCR analysis) of E-cadherin (*Cdh1*), vimentin (*Vim*), N-cadherin (*Cdh2*), Snail (*Snai1*) and Twist (*Twist1*) in clodrolip treated vs. control F9-tumors; n=5-6, bars: ± SEM. **B.** Western blotting analysis of E-cadherin, total β-catenin and active β-catenin expression in control and clodrolip treated F9-tumors; n=5. **C.** Quantification of B. Mean values in each group are indicated. **D.** Immunohistochemical analysis of $CD68^{+}$ macrophages (green), E-cadherin (red), β-catenin (red) and fibronectin (red) expression in a macrophage poor area (left panel) and a macrophage rich area (right panel) in a control F9-tumor section. The nuclei were stained with DAPI (blue). Scale bar=0.02 mm.

4.3.2

The macrophage secretome incites tumor cell invasion through induction of EMT *in vitro*

Having identified a correlation between TAM density and tumor cell EMT *in vivo*, 2D culture techniques were used to further characterize the mechanisms of action through which TAMs regulate this process. As the F9-teratocarcinoma cell line is highly sensitive to cytokine signaling *in vitro* as well as *in vivo*, it represents a potent cell culture model for EMT associated research (Krawitz and Kelly, 2008). In addition, the murine epithelial mammary gland NMuMG-cell line was used as it provides a model that is well characterized in the context of EMT (Bakin *et al*. 2000, Bhowmick *et al*. 2001, Gal *et al*. 2008, Vincent *et al*. 2009). The F9- and NMuMG-cells were cultured in conditioned medium generated by F9-cells (F9-CM) or NMuMG-cells (N-CM), or in medium conditioned by M2 polarized RAW264.7 macrophages (M-CM) (Wang *et al*. 2007). The cells were analyzed by immunofluorescence for morphology and cellular localization of E-cadherin, β-catenin, vimentin and fibronectin (Fig. 14A and 14E). Both cell lines displayed an epithelial morphology when cultured in F9-CM or N-CM. E-cadherin and β-catenin localized to the cellular membrane and vimentin and fibronectin were expressed at low levels. A similar profile was observed in cells cultured for 24 hours in M-CM. However, after long term culture in M-CM (7 days for F9-cells, 13 days for NMuMG-cells) the cells acquired an elongated fibroblast-like morphology. This morphological change correlated with partial loss of epithelial adherence junctions, reduced E-cadherin expression, cytoplasmic localization of β-catenin and increased levels of active β-catenin (Figs. 14B, 14F and suppl. Fig. S1). Moreover, the expression levels of vimentin and fibronectin were increased (Figs. 14A, 14E).

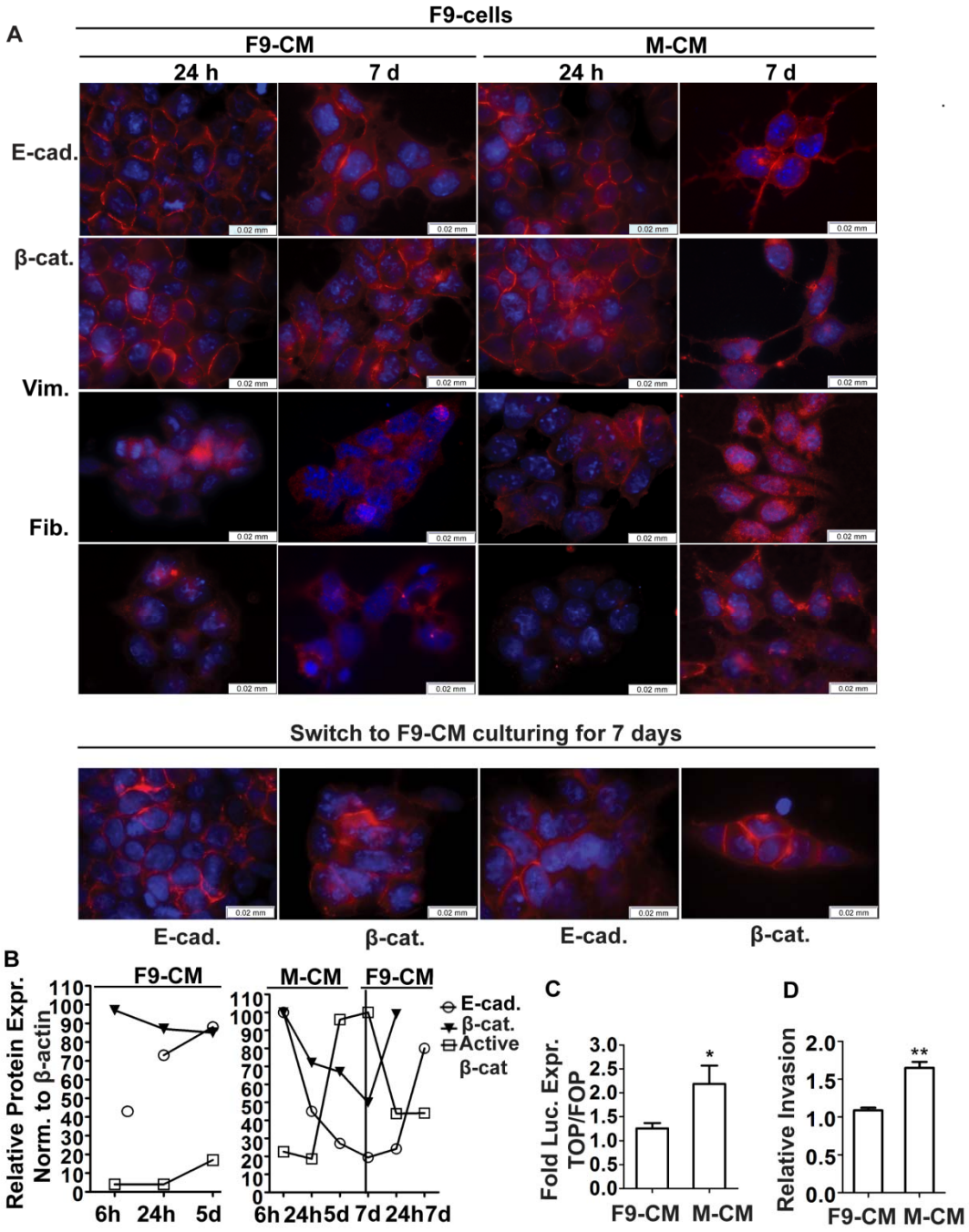

Fig.14. M-CM-induced EMT correlates with activation of the β-catenin pathway and increased invasion of F9-cells and NMuMG-cells *in vitro*. A. Immunofluorescence analysis of E-cadherin, β-catenin, vimentin and fibronectin expression and cellular localization in F9-cells cultured in F9-CM or M-CM for 24h and 7d. After 7d of culturing, the M-CM was substituted with F9-CM for another 7 days prior to immunohistochemical analysis of E-cadherin and β-catenin expression. Scale bar=0.02 mm. Proteins were stained red. Nuclei were stained with DAPI (blue). **B.** Protein expression (quantified from western blots shown in suppl. Fig.2) of E-cadherin and total β-catenin in F9-cells cultured in F9-CM or M-CM for 6h, 24h, 5d, and 7d. After 7d of culture, the F9-cells were cultured for another 7d in F9-CM. **C.** Fold luciferase expression (TOPFLASH/FOPFLASH) in F9- cells after 7d of culture in F9-CM or M-CM; n=7; * indicates $p < 0.05$. ** indicates $p<0.005$, unpaired *t*-test; bar=±SEM. **D.** Relative fold invasion by F9-cells in response to F9-CM or M-CM (48h, 1% Matrigel); n=8. Relative invasion was normalized to F9-CM or N-CM. ** indicates $p < 0.005$, unpaired *t*-test; bar=±SEM.

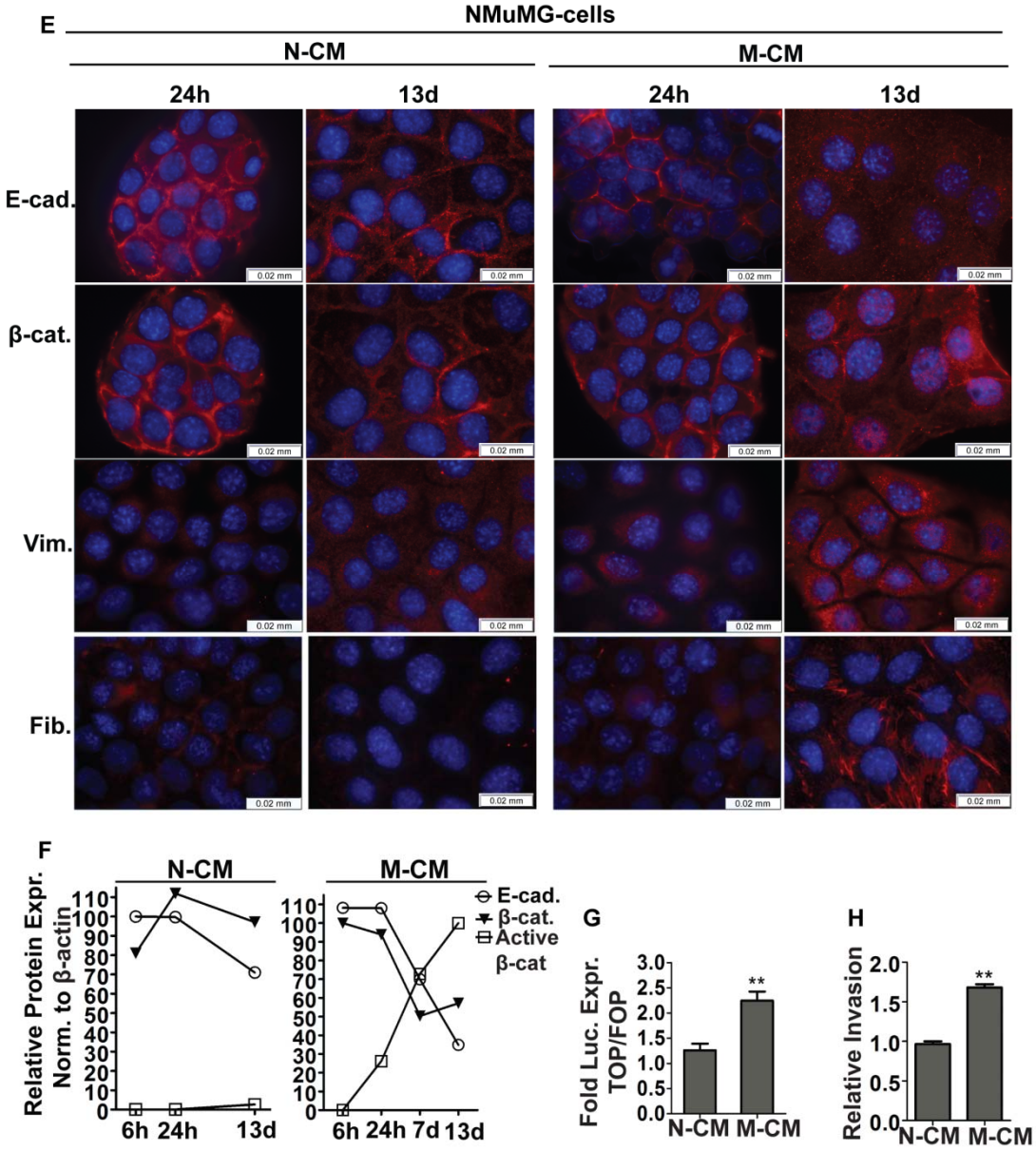

Fig.14 continued. E. Immunofluorescence analysis of E-cadherin, β-catenin, vimentin and fibronectin expression and cellular localization in NMuMG-cells cultured in N-CM or M-CM for 24h or 13d. Scale bar=0.02 mm. Proteins were stained red. Nuclei were stained with DAPI (blue). **F.** Protein expression (quantified from western blots shown in suppl. Fig.2) of E-cadherin and total β-catenin in NMuMG-cells cultured in N-CM or M-CM for 6 h, 24h, 7d and 13d. **G.** Fold luciferase expression (TOPFLASH/FOPFLASH) in NMuMG-cells after 13d of culture in N-CM or M-CM; n=7; * indicates $p < 0.05$. ** indicates $p<0.005$, unpaired *t*-test; bar=±SEM. **H.** Relative fold invasion by NMuMG-cells in response to F9-CM, N-CM or M-CM (48h, 1% Matrigel); n=3. Relative invasion was normalized to F9-CM or N-CM. ** indicates $p < 0.005$, unpaired *t*-test; bar=±SEM.

Interestingly, the phenotypic shift induced by M-CM was reversible in F9-cells. In a time depend manner, the expression levels of E-cadherin and membranous β-catenin were re-established and the level of active β-catenin was markedly reduced upon removal of M-CM (Figs. 11A lower panel and 14B left panel).

To confirm that the β-catenin pathway was transcriptionally activated upon release from the plasma membrane, the TOPFLASH/FOPFLASH reporter assay was implemented (Korinek *et al*. 1997). The TOPFLASH construct expresses luciferase downstream of a triple wild type Tcf-binding site, whereas the FOPFLASH construct is mutated at these binding sites and thus provides a negative control for the readout. Using this assay a significant increase in transcription activity of the β-catenin/Tcf complex in response to long term M-CM culturing was confirmed ($p<0.05$, unpaired *t*-test), (Fig. 14C and 14G).

One possible consequence of EMT is increased tumor cell invasion (Bakin *et al*. 2000, Lu *et al*. 2003, Nawshad *et al*. 2005, Yang and Weinberg, 2008, Thiery *et al*. 2009). Therefore, the invasive properties of F9- and NMuMG-cells were assessed in an *in vitro* invasion assay using Matrigel coated transwells. Both cell lines displayed significantly increased invasive properties in response to M-CM ($p<0.05$, unpaired *t*-test), (Fig. 14D and 14H). Thus, the M-CM induced mesenchymal phenotype correlated with an activation of the β-catenin pathway and increased invasive properties in both cell lines. As the cells never were in physical contact with macrophages, we concluded that the invasive, mesenchymal cell phenotype was regulated by soluble macrophage-derived cytokines.

4.3.3

Polarized macrophages regulate EMT in F9- and NMuMG-cells in a TGF β1 dependent manner

We next sought to identify the putative cytokine(s) involved in this process. The F9-tumors were evaluated for gene expression of four established inducers of EMT and invasion; *Wnt5a*, *Tgf-β1*, *Tgf-β2* and *Egf* (Gazit *et al*. 1999, Bakin *et al*. 2000, Bhowmick *et al*. 2001, Pukrop *et al*. 2006, Gal *et al*. 2008, Vincent *et al*. 2009). Whereas *Wnt5a* expression was too low for detection by q-PCR, *Egf* levels were ~ 3 fold decreased and *Tgf-β1* and *Tgf-β2* were ~ 2 fold decreased in clodrolip treated tumors relative to controls (Fig. 15A and suppl. Fig. S2). As TAMs are a main source of both EGF and TGF-β1 in the tumor microenvironment, these two candidates were further investigated (Yang *et al*. 2008, Yang and Weinberg, 2008). Both cell lines were cultured in DMEM medium +/- recombinant EGF (rEGF, 50 ng/ml) or DMEM medium +/- recombinant TGF-β1 (rTGF-β1, 2 ng/ml). The cells were analyzed by immunofluorescence for expression and localization of E-cadherin, β-catenin, vimentin and fibronectin (Fig. 15B and suppl. Fig. S3). Only rTGF-β1 induced a mesenchymal phenotype in the two cell lines. This phenotypic shift correlated with increased β-catenin/Tcf transcription activity (Fig. 15C). However, rEGF alone was sufficient to activate the MAP-kinase pathway downstream of the EGF-receptor in both cell lines, confirming that EGF receptors were functional (suppl. Fig. S4).

TGF-β mediates EMT partially through activation of the PI3K/Akt-pathway (Bakin *et al*. 2000, Nawshad *et al*. 2005). Western blotting analysis confirmed the activation of this pathway in F9-cells in response to 7 days of culture in DMEM +

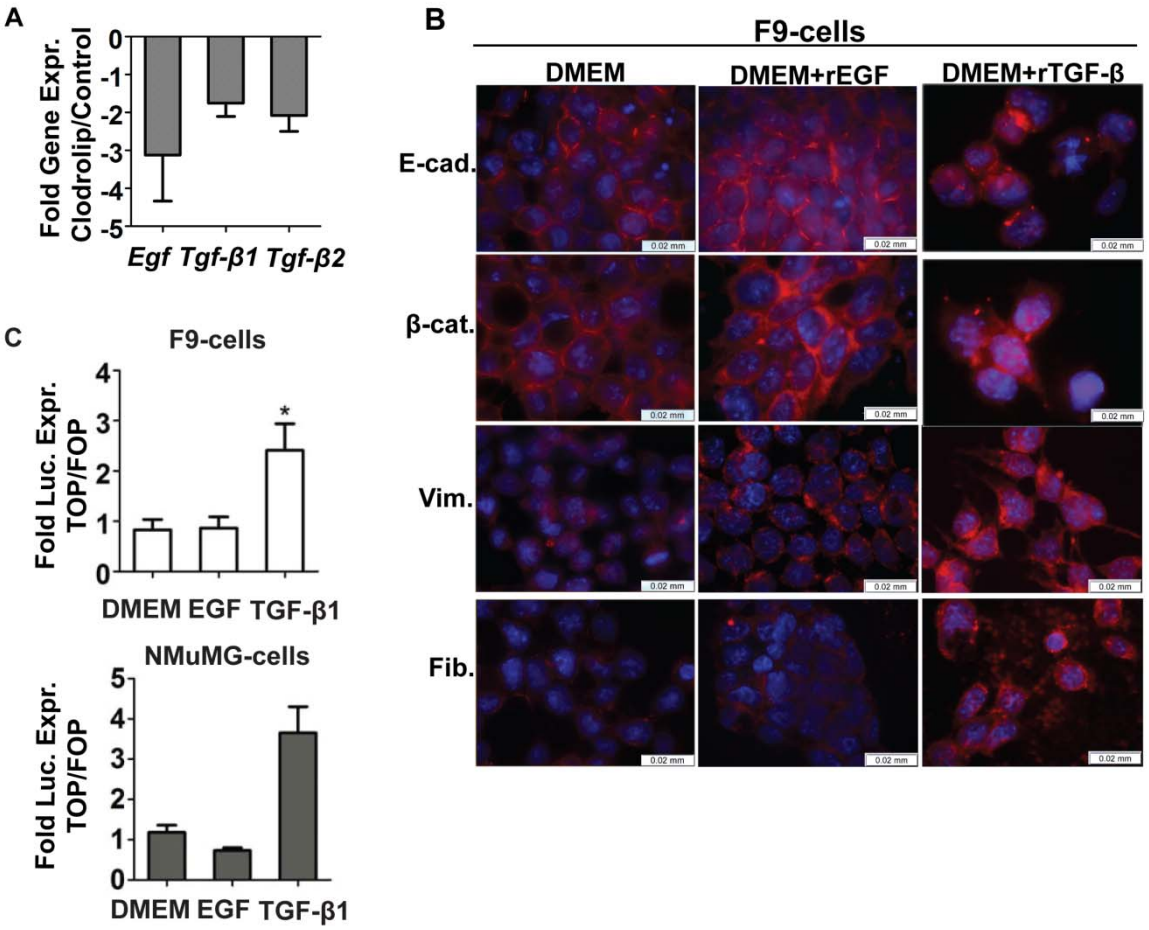

Fig.15. Recombinant TGF-β1 induces an EMT associated phenotype in F9- and NMuMG-cells *in vitro*. A. Relative fold gene expression (mRNA levels, qPCR analysis) of Egf, Tgf-β1 and Tgf-β2 in clodrolip treated vs. control F9-tumors; n=5-6, bars:±SEM. **B.** Immunofluorescence analysis of E-cadherin, β-catenin, vimentin and fibronectin expression and cellular localization in F9-cells cultured in DMEM +/- EGF (50 ng/ml) and DMEM +/- TGF-β1 (2 ng/ml) for 7d. Scale bar=0.02 mm. Proteins were stained red and nuclei were stained with DAPI (blue). **C.** Fold luciferase expression (TOPFLASH/FOPFLASH) in F9- (upper panel, n=5) and NMuMG-cells (lower panel, n=3) after 7d or 13d, respectively, of culture in DMEM+/- EGF or +/- TGF-β1. * indicates $p < 0.05$, unpaired *t*-test, bar=±SEM.

rTGF-β (Fig. 16A). Interestingly, M-CM activated this pathway in F9-cells as well, and the activation was abrogated when M-CM was neutralized for TGF-β (Fig. 16A). To test if neutralization of TGF-β was sufficient to abrogate M-CM induced EMT, F9- and NMuMG-cells were cultured in M-CM neutralized for TGF-β and subsequently analyzed for EMT marker expression by immunofluorescence. Both cell lines maintained an epithelial morphology and phenotype after 7 and 13 days of culture, respectively (Fig. 16B and suppl. Fig. S5). Concordantly, transcriptional activity of the β-catenin/Tcf complex resembled that of control cells (Fig. 16C). Lastly, TGF-β-neutralization significantly reduced the invasive properties of both cell lines in response to M-CM (Fig. 16D). However, when used as an exogenous chemoattractant TGF-β1 did not induce cell invasion (suppl. Fig. S6). Thus, macrophage-derived TGF-β regulated cell invasion through induction of an invasive cell phenotype.

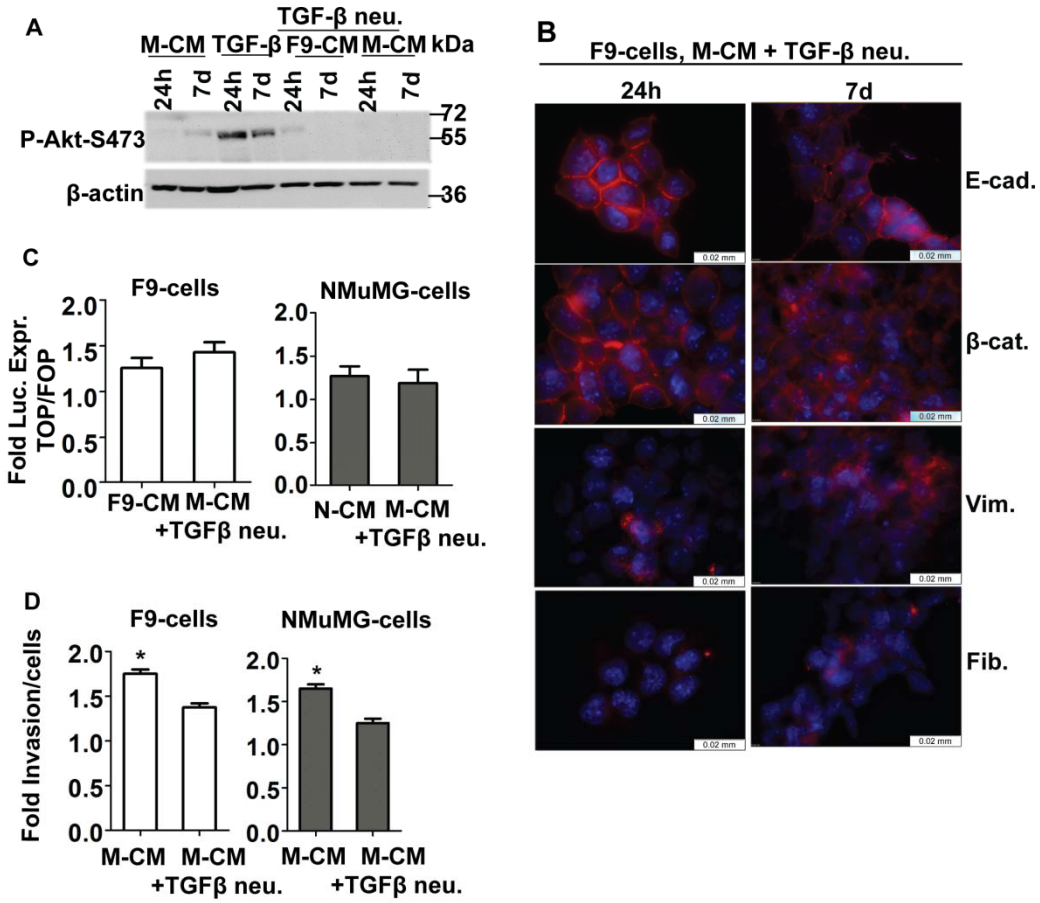

Fig. 16. Neutralization of TGF-β1 abrogates the M-CM induced EMT phenotype. A. Western blotting analysis of phospho-Akt (S473) in F9-cells after 24h and 7d of culture in M-CM, DMEM +/- TGF-β1, F9-CM and M-CM neutralized for TGF-β. **B.** Immunofluorescence analysis of E-cadherin, β-catenin, vimentin and fibronectin expression and cellular localization in F9-cells cultured for 24h and 7d in M-CM neutralized for TGF-β. **C.** Fold luciferase expression (TOPFLASH/FOPFLASH) in F9- (left) and NMuMG-cells (right) after 7d and 13d of culture in F9-CM, N-CM or M-CM neutralized for TGF-β, respectively; n=4, $p > 0.05$, unpaired *t*-test, bars=±SEM. **D.** Relative fold invasion by F9- (left) and NMuMG-cells (right) in response to M-CM +/- TGF-β neutralizing antibody (48h, 1% Matrigel), n=2, * indicates $p < 0.05$, unpaired *t*-test, bar=±SEM.

4.3.4

CD68$^+$ macrophage density correlates with mesenchymal tumor cell phenotype and tumor grade in NSCLC patients

Soltermann and collaborators have previously demonstrated a significant correlation between tumor cell expression of selected EMT-markers and various clinico-pathologic parameters for tumor progression in NSCLC patients (Soltermann *et al*. 2008). In this study we used NSCLC as a model to address the clinical relevance of intra-tumoral macrophages in tumor cell EMT. To this end, two independent researchers analyzed 491 NSCLC tissue samples of which 228 were adenocarcinomas (AC), 244 squamous cell carcinomas (SCC) and 19 adeno-squamous carcinomas (ASQ). Intra-tumoral macrophage density correlated significantly with tumor grade (Spearman correlation, SC>0.2), but not with other clinico-pathologic parameters for tumor progression like tumor stage (pT), lymph node metastasis/nodal stage (pN), and metastasis (pM), or tumor size (Table 2).

The tissue samples were further analyzed for tumor cell expression of EMTassociated markers (Table 3 and Fig. 17). Membranous E-cadherin and membranous β-catenin were chosen as epithelial markers, whereas cytoplamic vimentin, cytoplasmic periostin and cytoplasmic β-catenin were chosen as mesenchymal markers. Both evaluations established a moderate, positive correlation between intra-tumoral CD68$^+$ macrophage density and mesenchymal marker expression in tumor cells (SC>0.2). Moreover, CD68$^+$ macrophage density correlated negatively with membranous β-catenin in both evaluations (SC>-0.2). E-cadherin likewise showed a modest negative correlation with CD68$^+$ macrophage density in evaluation 1 (SC>-0.2), however, evaluation 2 could not confirm this correlation. Finally, tumor cell expression of mesenchymal markers correlated positively with tumor grade (SC<0.2). The data established a significant

and clinically relevant correlation between TAM infiltration, EMT associated tumor cell phenotype and tumor grade.

Table 2. Clinico-pathologic parameters and CD68$^+$ tumor infiltration

n=491	No.	%		CD68+, intra-tumoral Evaluation 1	CD68+, intra-tumoral Evaluation 2
Histotype					
AC	228	46.4			
SCC	244	49.7			
ASQ	19	3.9			
pT					
T1	100	20.4	SC	0.000	0.008
T2	268	54.6	*p*	0.993	0.859
T3	74	15.1			
T4	49	10.0			
pN					
N0	257	52.3	SC	-0.022	-0.072
N1	147	29.9	*p*	0.706	0.116
N2	77	15.7			
N3	10	2.0			
pM					
M0	448	91.2	SC	-0.117	-0.006
M1	43	8.8	*p*	0.082	0.161
Grade					
G1	28	5.7	SC	0.227	0.225
G2	253	51.1	*p*	*≤0.001**	*≤0.001**
G3	210	42.8			
Size					
≤ 3.7 cm	246	50.1	SC	0.072	0.080
> 3.7 cm	245	49.9	*p*	0.114	0.079

AC: Adenocarcinoma, SCC: Squamous cell carcinoma, ASQ: Ademosquamous carcinoma.
* indicates G1 and G2 versus G3.
Note: *p*-values and spearman correlation values (SC) are indicated.
Statistical analysis was performed in SPSS 16.0 for windows.

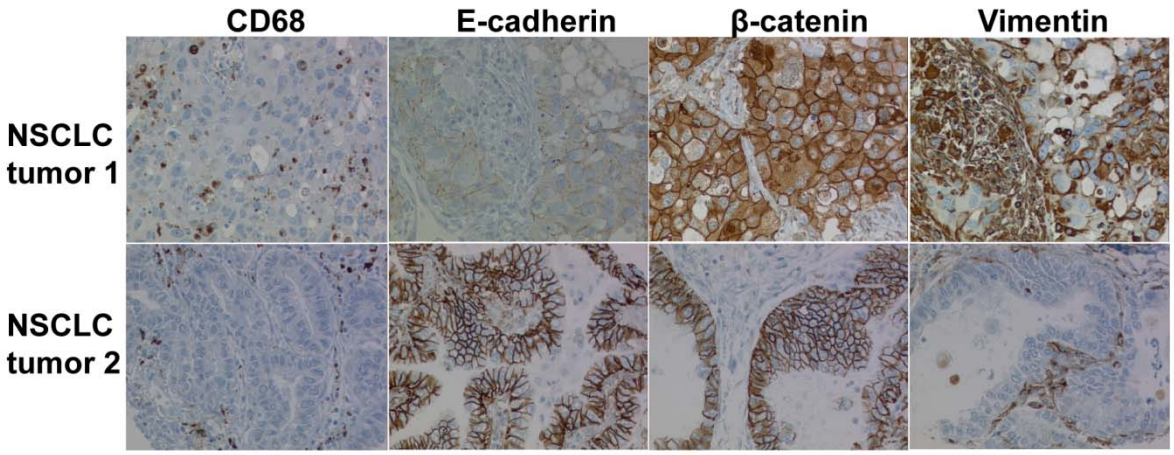

Fig.17. High intra-tumoral CD68^{+} macrophage density correlates with mesenchymal protein expression in NSCLC patient samples. NSCLC tissue sections stained for CD68, E-cadherin, β-catenin and vimentin. NSCLC tumor 1 is an adenocarcinoma of solid subtype, NSCLC tumor 2 is an adenocarcinoma of glandular subtype. NSCLC tumor 1 is a score 2 for CD68, score 1 for membranous E-cadherin, score 2 for membranous β-catenin, score 3 for cytoplasmic β-catenin and score 3 for vimentin. NSCLC tumor 2 is a score 1 for CD68, score 3 for membranous E-cadherin, score 3 for membranous β-catenin, score 1 for cytoplasmic β-catenin and score 0 for vimentin. Pictures were acquired with 20X magnification.

Table 3. EMT associated protein markers and CD68^{+} tumor infiltration

n=491		CD68+, intra-tumoral Evaluation 1	CD68+, intra-tumoral Evaluation 2	Grade 1-3
E-cadherin membrane	SC	-0.100	-0.073	-0.104
	p	*0.035*	0.111	*0.021*
Beta-catenin membrane	SC	-0.174	-0.175	-0.190
	p	*≤0.001*	*≤ 0.001*	*≤0.001*
Beta-catenin cytoplasm	SC	0.223	0.231	0.163
	p	*≤0.001*	*≤0.001*	*≤0.001*
Vimentin cytoplasm	SC	0.225	0.485	0.178
	p	*≤0.001*	*≤0.001*	*≤0.001*
Periostin cytoplasm	SC	0.177	0.215	0.112
	p	*≤0.001*	*≤0.001*	*0.008*

Note: *p*-values and spearman correlation values (SC) are indicated.
Statistical analysis was performed in SPSS 16.0 for windows

5 Discussion

5.1

Identifying novel implications of TAMs in tumor progression

The main focus of this study was to identify and characterize novel roles for TAMs in tumor progression. For this purpose, a micro array analysis of F9-teratocarcinomas infiltrated by – or depleted of – TAMs was performed. Depletion of TAMs was confirmed by a noticeable reduction in monocyte/ macrophage specific markers in clodrolip treated tumors. TAMs are well known regulators of tumor angiogenesis and ECM remodeling, thus to confirm the functional consequences of TAM depletion, genes involved in these processes were analyzed (Hagemann *et al*. 2004, Lin *et al.* 2006, Pollard, 2009, Gocheva *et al*. 2010). To this end, expression of genes involved in the angiogenic process and ECM remodeling was reduced as a consequence of clodrolip treatment.

A striking difference between the control and the clodrolip treated tumors was the expression of the epithelial marker E-cadherin, which correlated negatively with the expression of TAM markers. Loss of E-cadherin is a prerequisite for epithelial cell migration, and the micro array data revealed an inverse correlation between E-cadherin expression and expression of genes directly involved in the cytoskeleton changes required for successful migration, such as Arp2/3, CD44 and vimentin. It is of importance to note that macrophage migration is facilitated by similar molecular mechanisms, and the changes in expression of genes involved in this process can in part be due to the physical depletion of TAMs (Martise *et al*. 2009, Yilmatz and Christofori, 2009). Nevertheless, the observed reduction in expression of pro-migratory genes, in concert with the relative increase in E-cadherin and stathmin expression in TAM depleted tumors, suggested a potential

involvement of TAMs in regulation of pathways modulating epithelial cells migratory properties.

5.2

TAMs regulate EMT in epithelial tumor cells

Among the genes down-regulated in TAM depleted tumors were the mesenchymal markers vimentin and fibronectin as well as the CD44 receptor, which is an important mediator of cell invasion (Merzak *et al*. 1994, Korinek *et al*. 1997, van de Wettering *et al*. 2002, Marhaba and Zöller, 2004, Schmidt-Ott *et al*. 2007). Moreover, q-PCR analysis of the F9-tumors uncovered negative correlations between gene expression of E-cadherin and N-cadherin, snail and twist. The “cadherin switch” as well as the increase in mesenchymal gene expression and concordant up-regulation of snail and twist are molecular hallmarks of EMT (Maeda *et al*. 2004, Nawshad *et al*. 2005). Thus our data hint towards a link between TAMs and EMT mediated trans-differentiation in tumors cells.

Western blotting analysis of whole tumor extracts confirmed an increase in E-cadherin and total β-catenin levels in TAM depleted tumors relative to controls. As β-catenin can become transcriptionally active upon dissociation from E-cadherin, the levels of active β-catenin were tested by western blotting using an antibody specifically recognizing the active form of β-catenin (Korinek *et al*. 1997, Nawshad *et al*. 2005, Yilmatz and Christofori, 2009). However, the western blotting technique failed to confirm any correlation between E-cadherin and active β-catenin in whole tumor extracts. EMT is by nature transient, and the transcriptional onset of an EMT program is therefore difficult to trace in whole tumor extracts (Nawshad *et al*. 2005). Moreover, immunohistochemical analysis of frozen tumor

sections revealed a correlation between TAM density and mesenchymal tumor cell phenotype. This observation suggests that TAMs either recruit mesenchymal tumor cells or that they modulate the phenotype of the cells located predominantly in the neighboring microenvironment. Due to the rather small population of mesenchymal tumor cells, the fraction of active β-catenin may have been too low for detection by western blotting. The observation that TAM density correlated with a predominant mesenchymal tumor cell phenotype located in the neighboring environment suggests a causal relationship between tumor cell phenotype and TAM infiltration. TAMs are highly represented in the invasive front of solid tumors, thus the possibility that TAMs can modulate tumor cell phenotype locally can help to explain the poorly understood phenomenon of mesenchymal-like tumor cells often found to accumulate in the invasive tumor front (Brabletz *et al*. 2001, Franci *et al*. 2006, Sheehan *et al*. 2008, Joyce and Pollard, 2009).

TAMs ability to induce an EMT associated phenotypic shift was further investigated in F9- and NMuMG cells *in vitro*. In contrast to the heterogeneous tumor tissues, analysis of cell cultures confirmed a TAM dependent activation of the β-catenin pathway. This was further supported by immunofluorescence analysis, which revealed a macrophage culture medium (M-CM) dependent up-regulation of the β-catenin target genes, vimentin and fibronectin (Schmidt-Ott *et al*. 2007). Interestingly, the EMT transcription program, as well as the mesenchymal phenotype as evaluated by immunofluorescence, was reversible in F9-cells upon removal of M-CM. Mesenchymal cells of epithelial origin can re-acquire epithelial traits by undergoing MET. MET has been assigned with a hypothetical role in metastatic colony formation where plastic tumor cells in response to microenvironmental changes become epithelial and start colonizing the host tissue (Thiery, 2002, Hugo *et al*. 2007, Yang and Weinberg, 2008). MET

may also be of importance for local tumor invasion where tumor cells transiently acquire migratory properties to move across tumor boundaries and invade the hosting tissue (Thiery, 2002, Hugo *et al*. 2007). The reversibility of M-CM induced mesenchymal phenotype therefore suggests that TAMs contribute to tumor cell plasticity through regulation of EMT/MET.

5.2.1

TAMs signal EMT through TGF-β1

A number of *in vivo* studies have correlated TAM-infiltration into solid tumors with metastasis (Yang *et al*. 2008, Quian *et al*. 2009). In this respect, TAMs have been identified as a major source of chemokines that attract and recruit migratory tumor cells to distant sites (Wyckoff *et al*. 2004, Green *et al*. 2009). Concordantly, M-CM significantly stimulated the invasive properties of F9- and NMuMG-cells *in vitro*. In a candidate based screen, macrophage derived TGF-β was identified as the main cytokine inducing invasion in these cell lines, and neutralization of TGF-β1 in M-CM significantly reduced the invasive properties of the cells. However, rTGF-β1 alone did not prove to be a potent chemoattractant. These findings suggest that TGF-β regulates tumor cell invasion by induction of a chemotactic phenotype, rather than providing the chemoattracting signal itself. Immunofluorescence analysis of F9- and NMuMG-cells cultured in DMEM+/rTGF-β further support this notion, as rTGF-β potently induced a mesenchymal-like phenotype in the two cell lines. Collectively, the data demonstrate a direct involvement of TAMs in intrinsic regulation of invasive properties in tumor cells. TAMs are a significant stromal source of chemoattractants such as SDF-1, VEGF and EGF, and the data at present therefore suggest a model in which TAMs, through TGF-β signaling, can induce

an invasive phenotype via EMT, and concurrently provide chemokines to attract the invasive tumor cells (Fig. 18), (Wyckoff *et al*. 2004, Green *et al*. 2009).

TGF-β1 is a multifaceted regulator of EMT. On one hand, it directly induces EMT through activation of the TGF-β receptor. On the other, it stimulates the production of MMPs in an autocrine and paracrine fashion (Kim *et al*. 2007, Labbé *et al*. 2007, Uttamsingh *et al*. 2008). MMPs are important in this context as they are essential for proteolytic degradation of the ECM and thus contribute to increase the bioavailability of growth factors. Moreover, E-cadherin is a well established substrate for MMPs, and cleavage of E-cadherin can be sufficient to induce EMT (Radisky *et al*. 2005, Orlichenko and Radisky, 2008). The notion that TAMs induce EMT in a TGF-β dependent manner proposes that TAMs essentially orchestrate tumor cell invasion through activation of multiple EMT inducing pathways.

Although being a strong mediator of EMT, TGF-β often signals in conjunction with other cytokines such as Wnt and EGF *in vivo* (Nawshad *et al*. 2005, Labbé *et al*. 2007, Uttamsingh *et al*. 2008). It was therefore surprising to find that neutralization of TGF-β1 in M-CM was sufficient to abrogate the invasive, mesenchymal cell phenotype. Although synergistic effects between TGF-β1 and other macrophage-derived cytokines cannot be excluded, we can conclude that TGF-β1 is indispensable for the induction of EMT in F9- and NMuMG-cells. Whether TGF-β is indispensable for EMT induction *in vivo* is yet to be investigated.

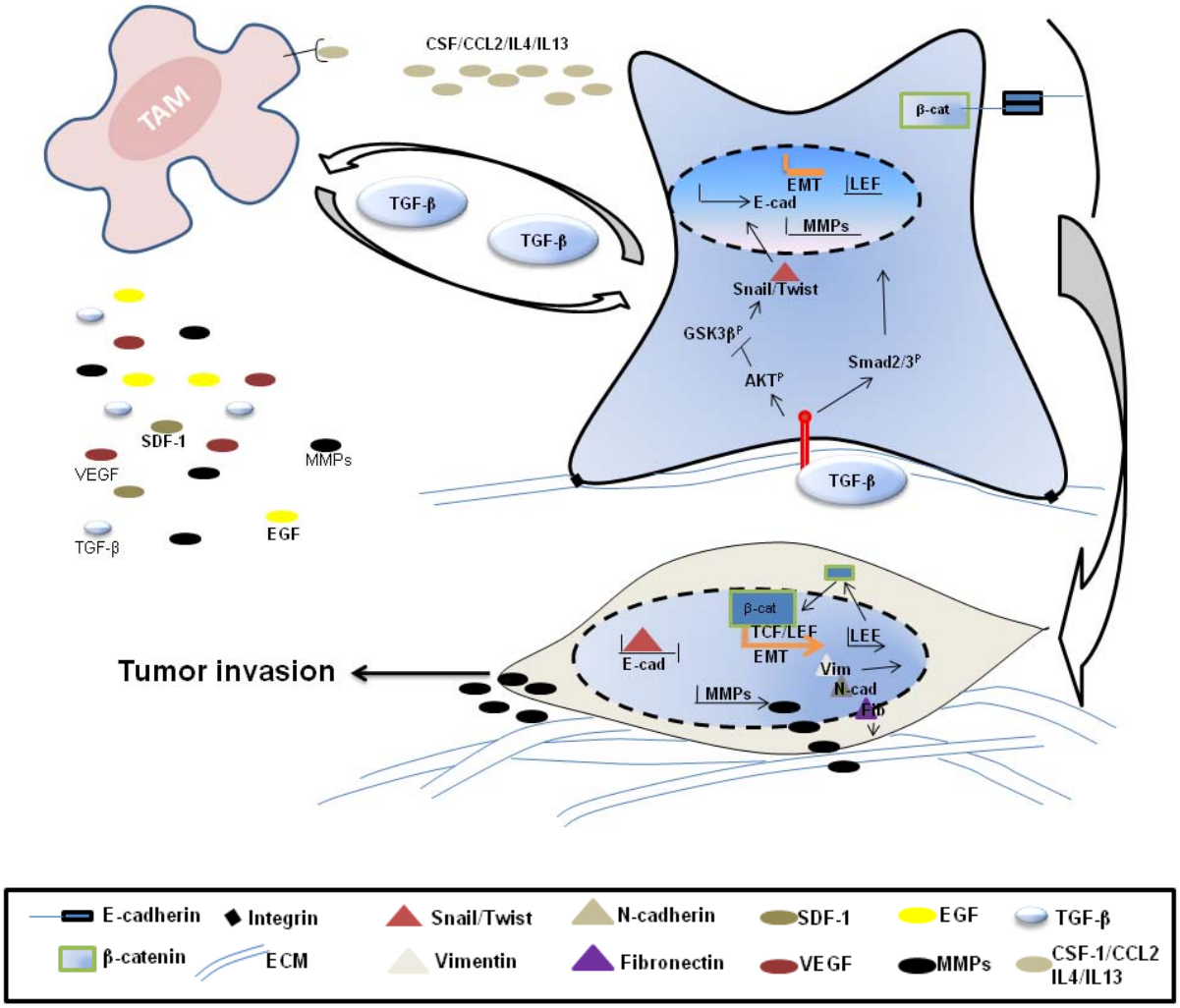

Fig. 18. Schematic illustration presenting the current model of TAM regulated tumor cell invasion. Epithelial cancer cells recruit and stimulate TAMs through cytokine signaling and TAMs respond by providing the microenvironment with TGF-β. TGF-β activates the TGF-β1/2 receptors in a paracrine fashion and through canonical and non-canonical signaling E-cadherin is suppressed, β-catenin is released and translocates to the nucleus to initiate the EMT transcription program. Consequently, the cytoskeleton is reorganized and the cell morphology changes from apicobasal to fibroblastoid. TGF-β furthermore stimulates the production of MMPs in a paracrine and autocrine fashion, and thus contributes significantly to the remodeling of the ECM and local invasion by tumor cells. Lastly, infiltrated TAMs can attract invasive tumor cells through secretion of chemokines.

5.2.2

TAM density correlates with mesenchymal tumor phenotype in NSCLC patients

An important aspect of this study was to address the clinical relevance of TAMs in regulation of tumor associated EMT. Soltermann and colleagues have previously proposed the mesenchymal proteins vimentin, periostin and the putative MET marker versican as prognostic markers for NSCLC (Soltermann *et al*. 2008). This was based on the observation that expression of these mesenchymal proteins in tumor cells correlated significantly with a number of clinico-pathologic parameters and disease outcome. In the current study, the correlation between intra-tumoral $CD68^+$ macrophage density and a mesenchymal tumor cell profile was evaluated. In concordance with the murine data, the NSCLC study revealed a positive, significant correlation between $CD68^+$ macrophage density and EMT-status of tumor cells in the local microenvironment. Moreover, both $CD68^+$ density and pronounced mesenchymal tumor profile correlated with tumor grade, confirming the clinical relevance of our *in vitro* and *in vivo* findings.

The correlation between mesenchymal tumor profile and tumor grade suggests a possible link between tumor de-differentiation and EMT-mediated trans-differentiation. Although being highly speculative, this potential connection should be further investigated as it may provide important information to increase the general understanding of the poorly understood de-differentiation process of epithelial tumors.

TAM infiltration and tumor cell EMT are generally associated with metastatic disease, however the data obtained in this study provide no evidence for such a correlation. It is here important to note that the metastatic process is complex

and not solely depends on these two factors (Yang and Weinberg, 2008, Joyce and Pollard, 2009).

5.3

Conclusions

The data presented here provide functional evidence for a regulatory role of TAMs in tumor cell EMT. Moreover, the identification of a correlation between intra-tumoral macrophage densities, EMT associated tumor profile and tumor grade in NSCLC patients confirms the clinical relevance of this finding.

Interestingly, recent reports have suggested that the mesenchymal tumor cell phenotype is related to the cancer stem cell phenotype and genomic instability, and it has been speculated that therapies abrogating EMT may lead to improved cancer management (Radisky *et al*. 2005, Mani *et al*. 2008, Thiery *et al*. 2009, Polyak and Weinberg, 2009). Data presented here contribute to increase our current understanding of the complex tumor-stroma interplay and provide novel insight valuable for the development of therapies abrogating EMT-associated disease progression.

5.4

Future perspective

Presently, the link between TAM-induced tumor cell EMT and invasion was investigated *in vitro*. Although the consequences of EMT in terms of invasion are generally accepted it is of great importance to confirm this link *in vivo* (Nawshad *et al*. 2005, Yang and Weinberg, 2008).

In this study I chose a subcutaneously implanted murine F9-teratocarcinoma model for *in vivo* investigations. Subcutaneous tumors generally lack invasive

fronts, and it is therefore impossible to draw substantial conclusions from these data regarding the role of TAM regulated EMT during local tumor invasion *in vivo*. This issue can be overcome by studying the phenomenon in orthotopic or spontaneously growing tumor models.

The murine, orthotopic 4T1 mammary carcinoma model is a highly invasive and metastatic model in which invasion was recently shown to be dependent on TGF-β signaling (Pulaski and Ostrand-Rosenberg, 2001, Fantozzi and Christofori, 2006, Bandyopadhyay *et al*. 2010). Due to its susceptibility to growth factor signaling, the orthotopic nature of the model and the fact that mammary tumors generally are infiltrated by TAMs, 4T1 may be a suitable model for such studies (Pulaski and Ostrand-Rosenberg, 2001, Leek and Harris, 2002). Moreover, this model forms spontaneous metastasis, which further facilitates investigations of the implication of TAMs and EMT in the metastatic process (Pulaski and Ostrand-Rosenberg, 2001, Fantozzi and Christofori, 2006, Bandyopadhyay *et al*. 2010).

The recent finding that EMT generates mesenchymal tumor cells with stem cell properties is very exciting (Mani *et al*. 2008, Polyak and Weinberg, 2009, Reiman *et al*. 2010, Santisteban *et al*. 2010). Cancer stem cells are highly drug resistant and they are believed to contribute significantly to patient relapse. It would therefore be interesting to perform a therapy study, in which TAM-depletion would be combined with a standard chemotherapy to test if TAM-depletion would increase the general tumor response to the treatment and prevent disease recurrence.

EMT is required for inflammation-induced cell migration and wound healing (Wu *et al*. 2009). This finding directly links the mechanism to pre-cancerous lesions, where EMT may be induced in response to pro-inflammatory signaling

(Wu *et al*. 2009, Toblar *et al*. 2010). Moreover, both EMT and inflammation have been linked to genomic instability, thus taken together, these independent studies suggests that EMT may contribute to the transforming events occurring during inflammation (Radisky *et al*. 2005, Colotta *et al*. 2009, Wu *et al*. 2009, Toblar *et al*. 2010). Although being highly speculative, this idea may provide an attractive model, in which therapeutic targeting of TAMs and/or their secretome may prevent transformation of pre-cancerous lesions. As this model is highly speculative, the possible link between TAM induced EMT and tumor initiation should be investigated carefully.

The identification of TAMs as stromal regulators of tumor cell EMT provide novel information to increase our understanding of the complex tumor-stroma crosstalk. However, it also raises new and interesting questions concerning the implications and regulation of TAMs and EMT in disease progression, and these questions should be addressed experimentally within the near future.

6. Materials and Methods

Antibodies and reagents

Primary antibodies were all anti-mouse: Anti-rat-E-cadherin (Abcam), anti-rabbit-β-catenin (Sigma), CD68-Alexa-488 (Serotec ABD), F4/80-Alexa-647 (AbD Serotec), anti-mouse-active-β-catenin (Millipore), anti-rabbit-β-actin (Abcam), anti-mouse-vimentin (Abcam), anti-rabbit-fibronectin (Abcam), anti-rabbit-AKT-phospho-S473 (Abcam). Secondary antibodies used: Goat-anti-rat-IgG-TRITC (Sigma), chicken-anti-rabbit-Alexa-594 (Molecular Probes), biotin-SP-Donkey-Anti-Rabbit-IgG, biotin-SP-donkey-anti-rat-IgG, and biotin-SP-donkey-anti-mouse-IgG (Jackson ImmunoResearch Laboratories). Streptavidin-HRP (Biolegends) was used to detect biotin labeled secondary antibodies. Recombinant TGF-β1 and TGF-β neutralizing antibody were purchased from R&D Biosystems and recombinant EGF was kindly provided by Dr. A. Mueller, IMCR, University of Zürich, Switzerland.

Cell lines and conditioned medium

F9-teratocarcinoma cells (ATCC CRL-1720) were grown on 0.01% gelatin. NMuMG-cells (CRL-1636) were kindly provided by Prof. G. Christofori, Center for Biomedicine, University of Basel, Switzerland. Both cell lines were cultured at 37°C, 5% CO_2, in DMEM/10% FBS/0.8% penicillin-streptomycin. RAW264.7 macrophages (Sigma) were cultured at 37°C, 5% CO_2, in RPMI1640/10% FBS/0.8% penicillin-streptomycin/1% Na-pyruvate (GIBCO). Conditioned medium was generated by culturing cells at 80% confluency in DMEM/10%FBS/ 0.8% penicillin-streptomycin for 24h. Collected conditioned medium was sterile filtered

prior to use (0.45µm pore size). RAW264.7 macrophages were polarized as previously described (Wang *et al*. 2007).

F9-tumors and macrophage depletion

F9-tumors were generated in female SV129S1 mice (Charles River, Germany) and liposomes were prepared as previously described (Zeisberger *et al*. 2006). Mice were kept in standard housing and normal diet at the animal facility of the University of Zürich. Animal studies were approved by the Veterinary Department of the Canton Zürich, Switzerland and performed under license 183/2006 issued to R.A. Schwendener. The control group (n=6) received empty liposomes (100 µl/20g body weight, i.p.), the test group (n=6) Clodrolip (1.5 mg clodronate/ 20g body weight, i.p.) starting 6h post tumor inoculation followed by the same dosage every 3rd day for 20d. Tumor size was measured with a caliper and volumes were calculated according to $V=\pi LW^2/6$ (L = largest tumor diameter, W = perpendicular diameter). Twenty four hours after the last i.p injection the study was terminated and tumors were collected for subsequent analysis Tumors subjected to immunohistochemistry and protein chemistry were stored in Hanks salt buffer (GIBCO) at -80°C. Tumors subjected to q-PCR were stored in RNAlater as described by the provider (Qiagen).

Immunohistochemistry of frozen F9-tissue sections

Frozen sections (8 µm thickness) were acetone fixed, blocked with 1% BSA/TBS, incubated with primary and secondary antibodies (diluted as recommended by the provider) overnight at 4°C. Nuclei were stained with DAPI (1 µg/ml). The sections were mounted with Vectashield (Vector labs) and visualized with an Olympus fluorescence microscope (1X81) using the CellR software

(Olympus). Pictures were acquired with 1x1 binning and 15x or 150x magnification at exposure times of 49-56 ns for DAPI and 500-550 ns for TRITC, Alexa-488, Alexa-594 and Alexa-647. The pictures were merged in Adobe Photoshop CS4.

In vitro induction of EMT and immunofluorescence analysis of F9- and NMuMG-cells

F9- and NMuMG-cells were cultured on sterile glass coverslips in F9-CM or N-CM, M-CM +/- TGF-β neutralizing antibody (30 ng/ml), DMEM/10%FBS +/- rEGF (50 ng/ml) or +/- rTGF-β1 (2 ng/ml). The medium was renewed every 24h. The cells were harvested at the time points annotated, fixed with 3% formaldehyde, stained and visualized as described for frozen sections.

In vitro invasion assay

The cells were starved in serum free medium for 6h and seeded (100.000 cells/well) in Boyden chambers (Corning, 8 µm pore size) coated with 50µl 1% Matrigel (BD Biosciences). F9-CM or N-CM and M-CM +/-TGF-β neutralizing antibody (30 ng/ml) were used as chemoattractants. The assay was incubated for 48h at 37°C, 5% CO_2. The relative number of invading cells was estimated by resazurin live cell detection using providers protocol (Invitrogen). The relative fold invasion was normalized to control conditions.

Live cell viability assay

Tumor polarized RAW 264.7 (IL-4/IL-13 polarized as described by Wang *et al*. 2007) cells were seeded in 96 well plates (10`000 cells/well) and grown under standard conditions for 24 hours. The growth medium was renewed and varying concentrations of Clodrolip or empty liposomes (0-16 mM) were added directly into

the medium. The cells were cultured for additional 24 hours, washed, and the relative number of live cells was estimated by resazurin live cell detection using providers protocol (Invitrogen).

Microarray preparation and analysis

Total mRNA was isolated from homogenized F9-tumors using the RNAeasy kit (Qiagen). The quality of the mRNA was tested using the Experion RNA Std Sense Analysis kit/Bioanalyser following the providers protocol (Bio-Rad). Samples that showed good quality were submitted to the Functional Genomics Center Zurich, University of Zurich, Switzerland, under the project number 175, for microarray chip analysis (Affymetrix). Target genes exceeding a threshold of > 2 fold +/- were selected and screened for pathway-involvement using ermine 2.1.1.5 (free software from Columbia University, NY, USA).

NSCLC tissue microarrays and patient cohort

The selection of NSCLC patient tissue samples and generation of the tissue microarrays (TMAs) were done as previously described (Soltermann *et al*. 2008). In brief, formalin-fixed and paraffin-embedded tumor tissue samples of 532 NSCLC patients were reviewed by two pathologists and two representative tissue cores (0.6 mm) were assembled into 3 TMAs. Patients having obtained neo-adjuvant chemotherapy were excluded. Sarcomatoid carcinomas were excluded from this study and EMT was strictly defined by expression of EMT-associated protein markers and not by morphology (number of final samples = 491). The study was approved by the institutional review board of the University Hospital Zürich under reference number StV-29-2009.

NSCLC, immunohistochemistry

Immunohistochemistry on 4 μm sections from the TMA blocks was performed using automated platforms from either Ventana (Ventana Medical Systems) or Bond (Vision Biosystems). Following primary monoclonal antibodies were used: anti-CD68 (DAKO-Cytomation, clone PG-M1, 1:50 dilution), anti-E-cadherin (Cell Marque, clone EP700Y, 1:200), anti-β–catenin (BD Transduction laboratories, clone 14, 1:50), anti-vimentin (DAKO-Cytomation, 1:250) and Pab anti-periostin (BioVendor, 1:500). Detection was performed using the UltraVIEW-DAB (Ventana) or the Refine-DAB (Bond) detection kits, including respective secondary antibodies as described before (Soltermann *et al*. 2008).

NSCLC, interpretation and statistical methods

Distinct intra-epithelial $CD68^+$ macrophage density was semi quantitatively scored by AKB and by two pathologists (VT and AS) on a multi-headed microscope (Zeiss Axioscope 2 MOT) using a four-tiered system: 0 (negative), 1+ (few to some $CD68^+$ macrophages), 2+ (moderate number of $CD68^+$ macrophages), and 3+ (multiple $CD68^+$ macrophages). Membranous β-catenin (AKB and VT) and membranous E-cadherin (VT and AS) were evaluated for staining intensity according to a four-tiered system: 0 (negative, no detectable staining), 1+ (weak, faint discontinuous membrane staining), 2+ (moderate and continuous membrane staining), 3+ (strong and continuous membrane staining). Cytoplasmic β-catenin (AKB and VT), cytoplasmic vimentin (AKB & AS) and cytoplasmic periostin (VT & AS) were scored due to staining intensity: 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). The statistical analysis was performed with the SPSS 16.0 software for Windows (IBM).

Quantitative real time PCR

RNA was isolated from F9-tumors as described elsewhere. cDNA was synthesized using the Omniscript reverse transcriptase kit (Qiagen). Q-PCRs were carried out using the LightCycler 480 instrument (Roche Diagnostics). PCR program: 95ºC, 5 min, 45 cycles of 10 s 95ºC, 25 s annealing and 15 s 72ºC. Primers were obtained from Microsynth, Switzerland, (primer sequences and annealing temperatures, see supplementary Table S1). The PCR-products were analyzed on 1.5% agarose gels. Expression of all target genes was normalized to β-actin and GAPDH levels. All samples were run in duplicates of n=5-6/per group. Fold change was calculated using the Pfaffl equation (Pfaffl, 2001).

TOPFLASH reporter assay

The TOPFLASH reporter assay was established as previously described (Korinek *et al*. 1997). The fold value was calculated as TOPFLASH/FOPFLASH, where TOPFLASH is the plasmid expressing luciferase downstream of three wild type β-catenin/Tcf binding sites, and FOPFLASH is the plasmid with mutated binding sites. Renilla pRL SV40 was included as transfection control. Luciferase was detected using the Dual Glo Luciferase detection kit (Promega). The samples were analyzed an SPECTRAmax® GEMINI XS instrument (Molecular Devices). The cells were transfected two days prior to luciferase readout using a standard in-house transfection protocol.

Western blots

F9- and NMuMG-cells were lysed (1% NP-40, 100 mM ortho vanadate, 100 mM 3-indoleacetic acid (IAA), 100 mM phenylmethylsulfonylfluoride (PMSF)) at annotated time points and snap frozen in liquid nitrogen. Frozen tumors were

soaked in lysis buffer and homogenized using an Ultra Turrax T8 homogenizer (IKA-Werke). Protein concentration was determined by Bradford analysis (Biorad). The blots were quantified using ImageQuant5.2 software (Amersham Biosciences). Protein expression was normalized to β-actin.

Graphs and statistical calculations were performed with the GraphPad Prism 5 software unless otherwise noted.

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8. List of abbreviation

APC: Adenomatosis polyposis coli
Arp2/3: Actin related protein 2/3 complex
ATP: Adenosine triphosphate
bHLH: Basic helix-loop-helix
CAF: Cancer associated fibroblasts
COX-2: Cyclo-oxogenase 2
CSF-1: Colony stimulating factor-1
CSFR-1: Colony stimulating factor receptor-1
ECM: Extra cellular matrix
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
EMT: Epithelial to mesenchymal transition
F9-CM: F9-cell conditioned medium
GSK3β: Glycogen synthase kinase-3β
HIF-1: Hypoxia inducible factor-1
HGF: Hepatocyte growth factor
iNOS: Inducible nitric oxide synthase
INF-γ: Interferon-γ
LPS: Lipo-polysaccharide
M1: Pro-inflammatory macrophages, M1 phenotype
M2: Pro-tumor macrophages, M2 phenotype
M-CM: Macrophage conditioned medium
MET: Mesenchymal to epithelial transition
MMP: Matrix metalloproteinase
N1: Pro-inflammatory neutrophils, N1 phenotype
N2: Pro-tumor neutrophils, N2 phenotype
N-CM: NMuMG-cell conditioned medium
NK-cells: Natural killer cells
NFκB: Nuclear factor-κB
PI3K: Phosphoinositide-3-kinase
PLC: Phospholipase Cγ
q-PCR: Quantitative polymerase chair reaction
ROI: Reactive oxygen intermediates
ROS: Reactive oxygen species
RTK: Receptor tyrosine kinase
SC: Spearman correlation
SDF-1: Stromal derived factor-1
SEM: Standard error of mean
TAM: Tumor associated macrophage

TGF-β: Transforming growth factor-β

TGFR-β: Transforming growth factor-β receptor

TNF-α: Tumor necrosis factor-α

T_{regs}: T-regulator cells (Th_2 response)

uPA: Uro-kinase plasminogen activator

uPAR: Uro-kinase plasminogen activator receptor

VEGF: Vascular endothelial growth factor

9. Gene list

Arp2/3: Actin related protein 2/3 complex

Cd44: CD44

Cd68: CD68

Cdh1: E-cadherin

Cdh2: N-cadherin

Ctsb: Cathepsin B

Ctsh: Cathepsin H

Col18a1: Procollagen 18

CSFR-1: Colony stimulating factor receptor-1

Egf. Epidermal growth factor

Erdnrb: Endothelin receptor B

Fn: Fibronectin

Lmna: Lamin A

Lamb: Laminin b

Mmd : Monocyte to macrophage differentiation factor

MMP14: Matrix metalloproteinase 14

Mrc : Mannose receptor C

Tgfβ1,2: Transforming growth factor 1 and 2

Thbs1: Thrombospondin 1

Twist1: Twist

Snai1: Snail

Stmn1: Stathmin 1

Vim: Vimentin

Wnt5a: Wnt 5a

Supplementary figure S1

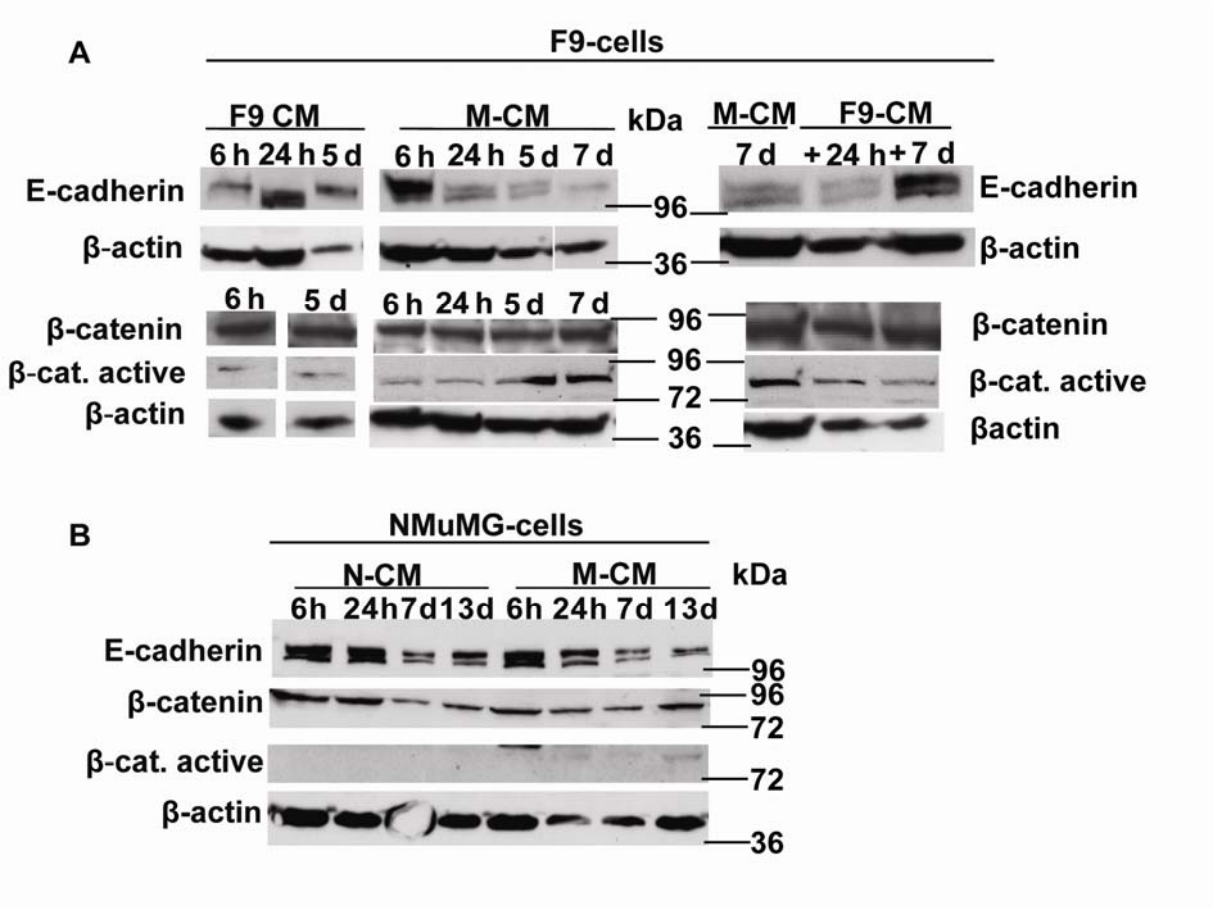


Fig. S1. Protein expression analysis of F9- and NMuMG-cells cultured in F9/N-CM and M-CM. A. Western blotting analysis of E-cadherin, β-catenin and active β-catenin expression by F9-cells cultured in F9-CM or M-CM. **B.** Western blotting analysis of E-cadherin, β-catenin and active β-catenin expression by NMuMG-cells cultured in N-CM or M-CM

Supplementary figure S2

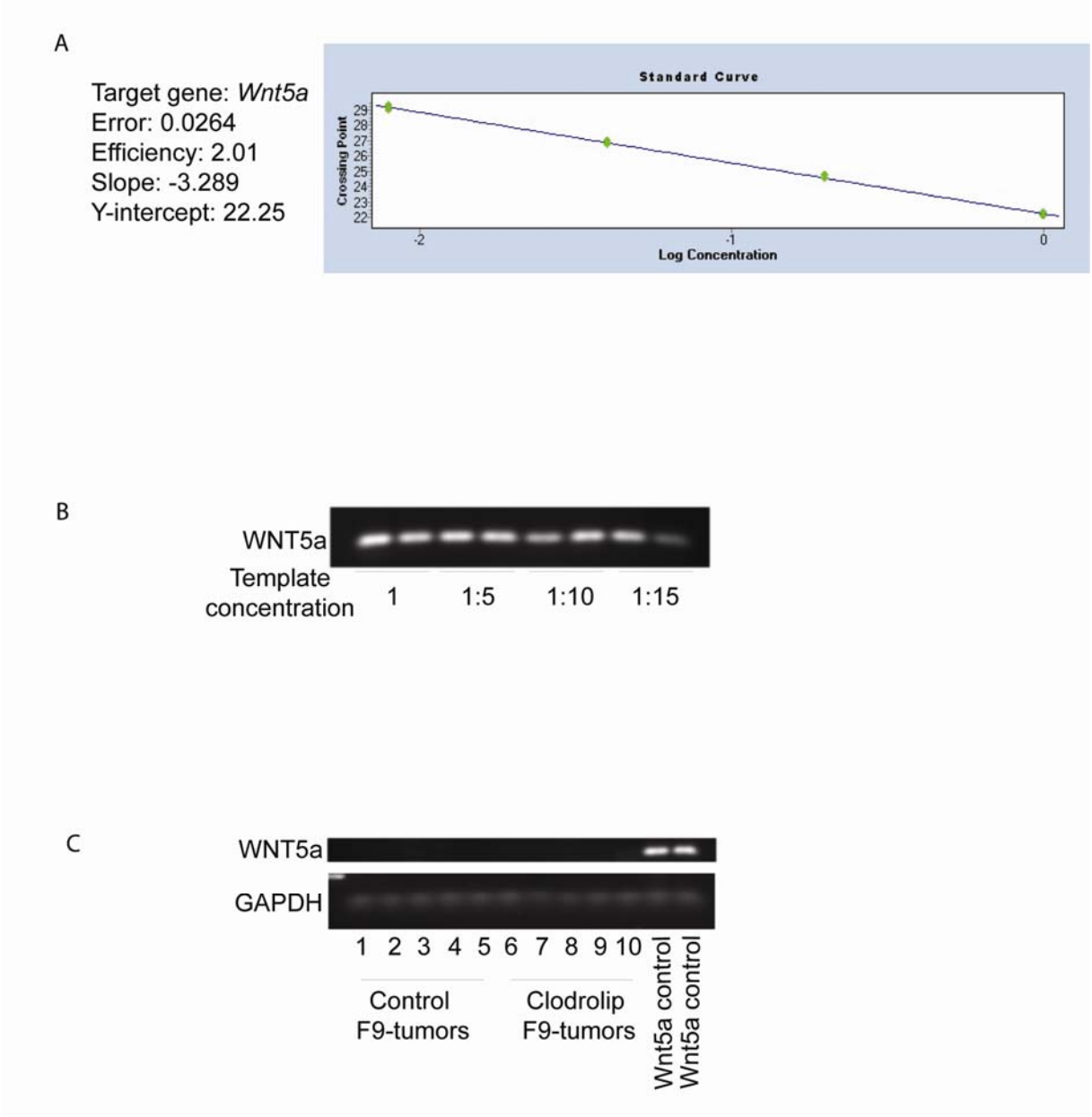


Fig. S2. *Wnt5a* gene expression in F9-teratocarinomas. A. Standard curve generated from 4 dilutions of a *Wnt5a* overexpressing cDNA template. **B.** PCR products from a *Wnt5a* template, evaluated on a 1.5% agarose gel (dilutions: 1, 1:5, 1:10 and 1:15). **C.** PCR products obtained from the reaction using template from 5 control F9-tumors, 5 clodrolip treated F9-tumors and 2 *Wnt5a* overexpressing control templates. GAPDH primers were included in the reaction as control for the reaction and gel loading.

Supplementary figure S3

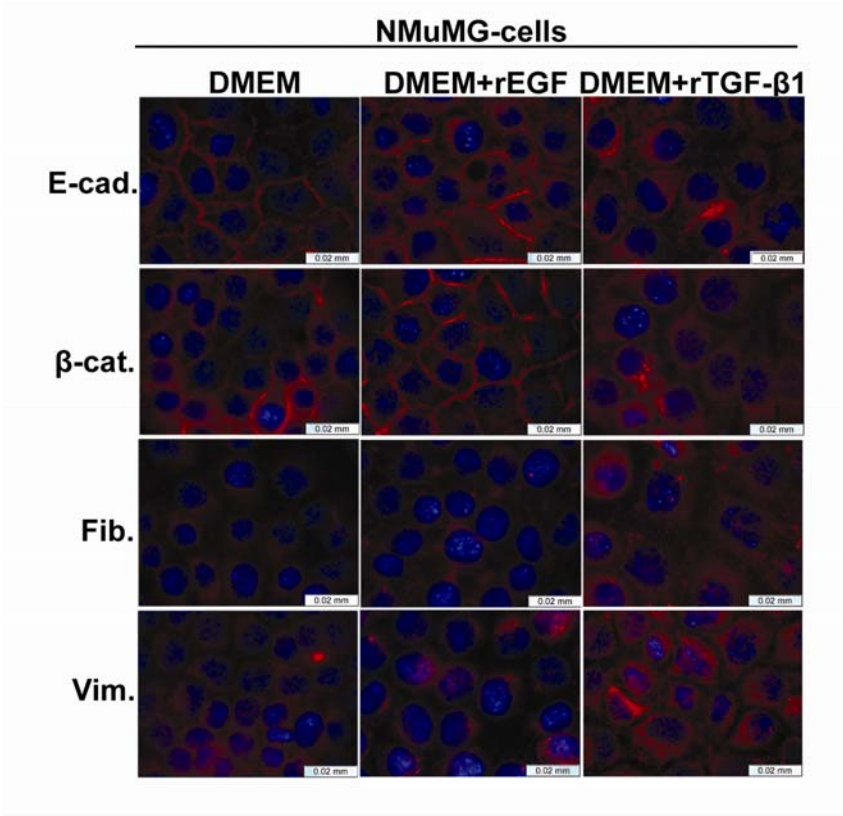


Fig. S3. rTGF-β1 induces an EMT phenotype in NMuMG-cells *in vitro*. Immunofluorescence analysis of E-cadherin, β-catenin, vimentin and fibronectin (all in red) expression in NMuMG-cells cultured in DMEM +/- rEGF (50ng/ml) or DMEM +/- rTGF-β1 (2ng/ml) for 13 days. Scale bar=0.02 mm. Nuclei were stained with DAPI.

Supplementary figure S4

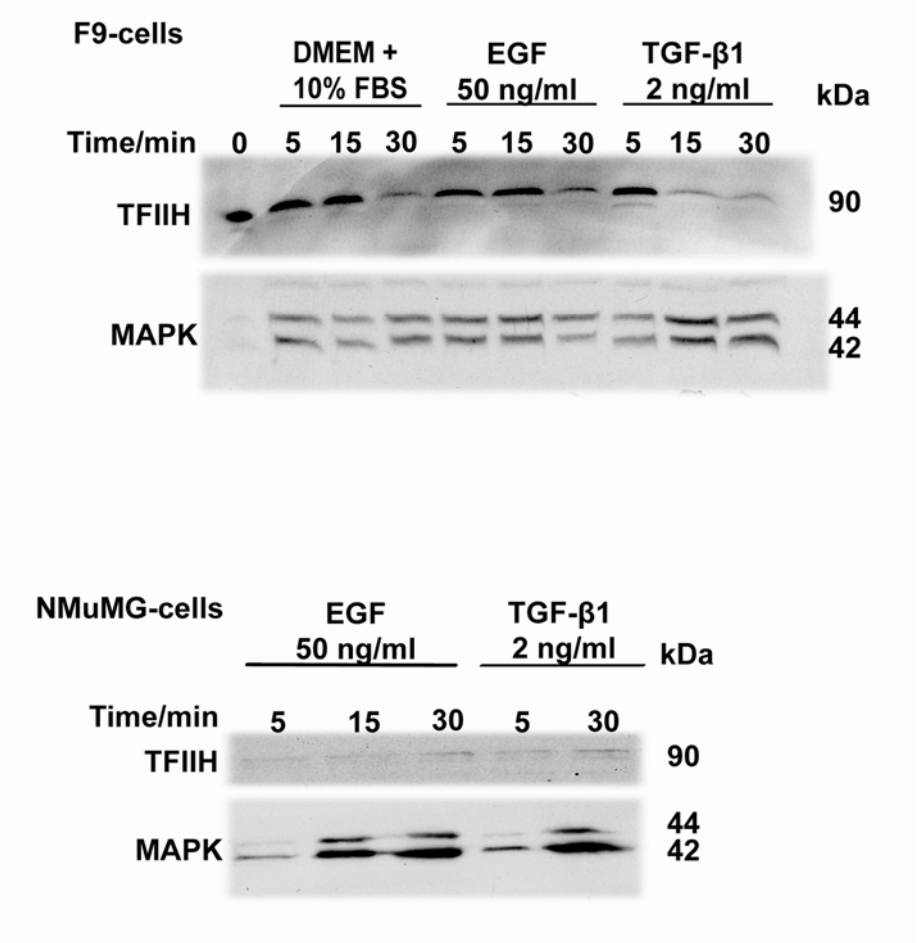


Fig. S4. Recombinant EGF and TGF-β1 activates the MAPK pathway in F9- and NMuMG-cells. Western blotting analysis of MAPK activation (MAPK 42/44) in F9- and NMuMG-cells after stimulation with rEGF (50ng/ml) and rTGF-β1 (2ng/ml) for 5, 15 and 30 min. Transcription Factor II H (TFIIH) was included as loading control.

Supplementary figure S5

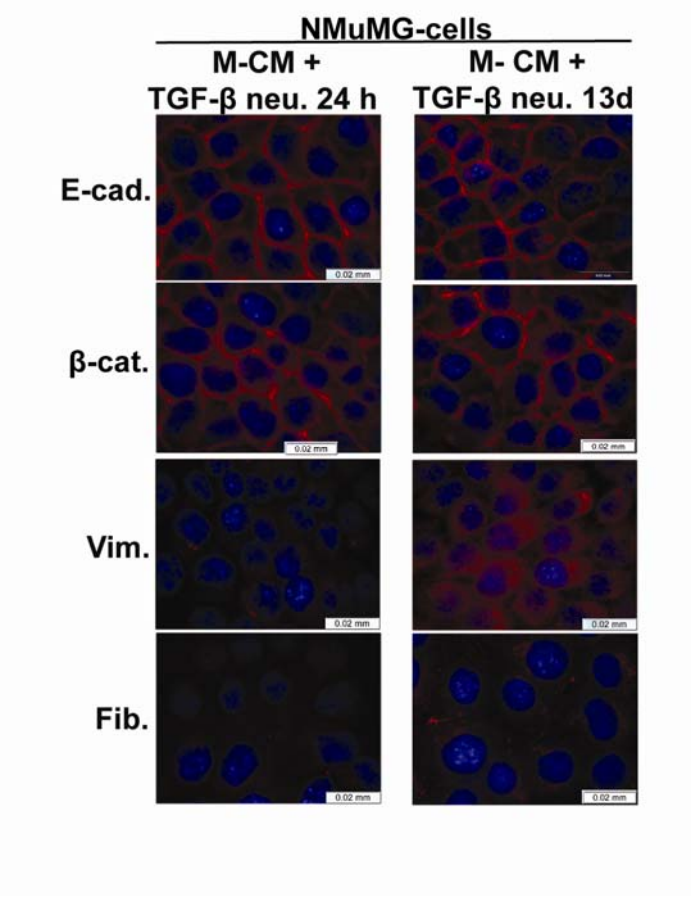


Fig. S5. Neutralization of TGF-β abrogates M-CM induced EMT *in vitro*. Immunofluorescence analysis of E-cadherin, β-catenin, vimentin and fibronectin (all in red) expression in NMuMG-cells cultured for 24 hours and 13 days in M-CM neutralized for TGF-β. Scale bar=0.02 mm. Nuclei were stained with DAPI.

Supplementary figure S6

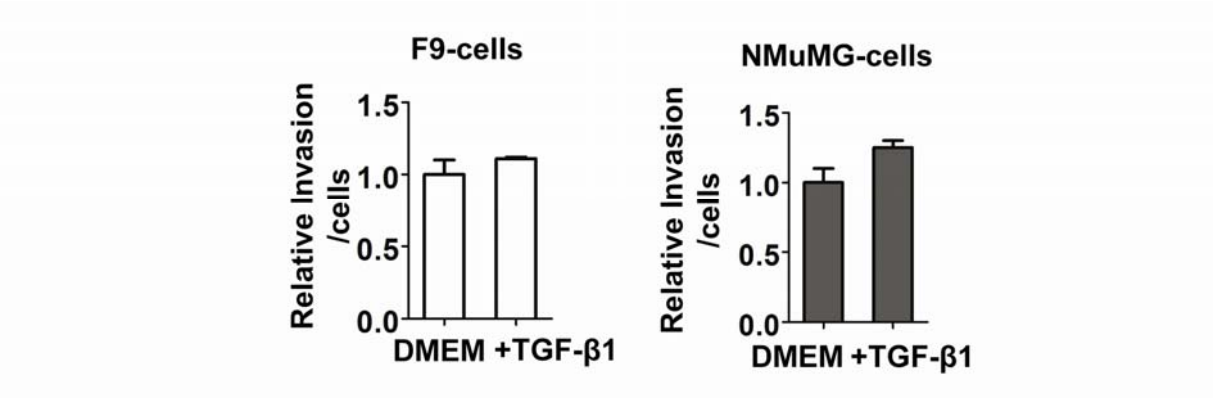
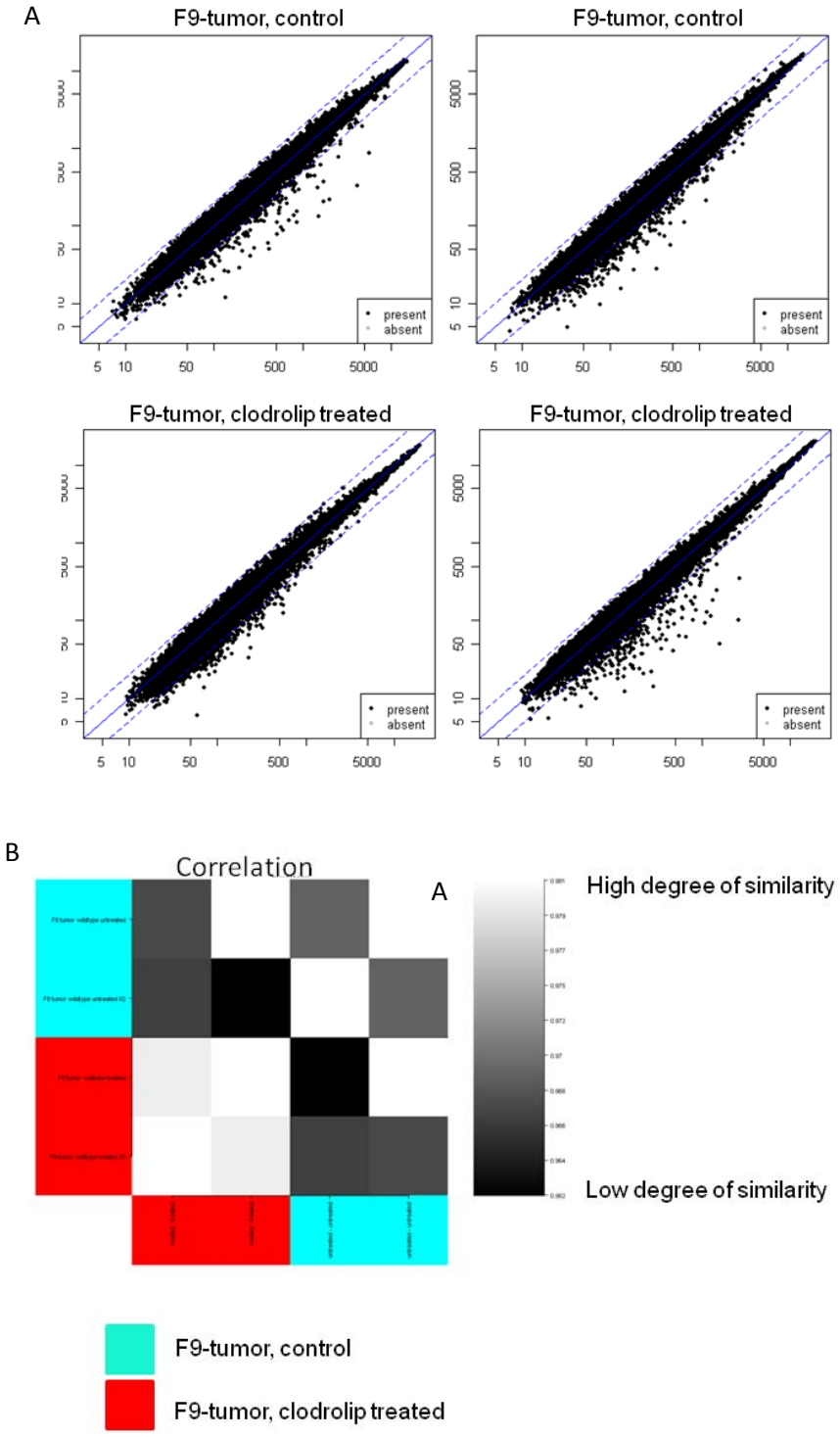


Fig. S6. rTGF-β1 is not a potent chemoattractant for F9- and NMuMG-cells *in vitro*. Fold invasion of F9- (left) and NMuMG-cells (right) in response to DMEM +/- rTGF-β (2ng/ml), (48 hours, 1% Matrigel); n=2, $p > 0.05$, unpaired *t*-test, bar = ± SEM.



Appendix 2. A. Quality control of mRNA isolated from two control (upper panel) and two clodrolip treated (lower panel) F9-teratocarcinoas (QC was performed using the Experion RNA Std Sense Analysis kit/Bioanalyser). **B.** Correlation plot of the four F9-tumors (two controls, two clodrolip treated) included in the final micro array analysis. Dark squares indicates low similarity in gene expression between the tumors, and light squares indicates high similarity in gene expression.

Title: Tumor Associated Macrophages Regulate Epithelial-Mesenchymal Transition in Tumor Cells in a TGF-β-dependent Manner.

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Running Title: TAMs regulate tumor EMT in a TGF-β-dependent manner

Corresponding Author: Schwendener, Reto A.

Key Words: Tumor-associated Macrophages, Tumor progression, Tumor invasion, Epithelial-Mesenchymal Transition, TGF-β.

Grant Support: Swiss National Science Foundation (SNF) (AKB), Stiftung zur Krebsbekämpfung (AKB), Fondation Nuovo-Soldati (VT).

Abstract:

Purpose: Tumor progression is highly influenced by stromal components. Several stromal-derived factors contribute to tumor progression by inducing epithelial-mesenchymal transition (EMT). As EMT promotes tumor cell invasion, therapies abrogating it can significantly improve cancer management. Here, we characterize tumor associated macrophages (TAMs) as key stromal regulators of tumor cell EMT *in vivo* and *in vitro*.

Experimental design: Tumor cell expression of EMT-associated markers was analyzed in murine F9-teratocarcinomas infiltrated by - or depleted of - TAMs. *In vivo* depletion of TAMs was performed using clodronate-liposomes. TAM-induced EMT was further characterized in two murine cell lines, the F9- and the mammary gland NMuMG cell line, using a conditioned medium culture approach. The clinical relevance of our findings was evaluated in a tissue microarray platform representing 491 NSCLC patients.

Results: Gene expression analysis of F9-teratocarcinoma tumors revealed a negative correlation between TAM-depletion and mesenchymal marker expression. *In vitro*, long term exposure of F9- and NMuMG-cells to macrophage-conditioned medium led to decreased expression of the epithelial adherence protein E-cadherin, activation of the EMT-mediating β-catenin pathway, increased expression of mesenchymal markers and increased invasive properties. In a candidate based screen, macrophage-derived TGF-β was identified as the main inducer of this EMT-associated phenotype. Lastly, histopathologic evaluation of 491 NSCLC patient samples identified a positive correlation between intra-tumoral TAM densities, mesenchymal tumor phenotype and high tumor grade.

Conclusions: Data presented here identify TAMs as key regulators of EMT-promoted tumor progression and contribute novel information to the discussion on therapeutic targeting of the tumor stroma.

Acknowledgement

Initially, I would like to thank Prof. Dr. Reto Schwendener, for offering me a position as PhD student in his laboratory, for supporting me on my choice of project, its continuous development, and for giving me a professional reason to move to Switzerland. The years in your laboratory have been exciting, challenging and motivating.

Also, I would like to thank Prof. Dr. Josef Jiricny, Director of the Institute of Molecular Cancer Research, for agreeing to be my “Doctor father” and thereby becoming a valued member of my committee. In my committee I have also have had the pleasure of having Prof. Dr. Urs Greber, Institute of Zoology, University of Zurich, and Prof. Dr. Curzio Ruegg, Faculté de biologie et de medicine, University of Lausanne, currently moving to Friburg University. I have benefited greatly from your scientific input, discussions and suggestion. It has been a pleasure to get to know you, and to work under your supervision.

Thanks to Dr. Sushil Kumar and soon to be Dr. Sibel Mete for support, scientific (and private) discussions, smiles and coffees. A special thanks goes to Sushil for sharing his unlimited knowledge on cellular signaling, a discipline I have learned to appreciate.

This work was partially done in collaboration with Alex Soltermann, MD and Verena Tischler, MD, Institute for Surgical Pathology, Department of Pathology, University Hospital Zurich. Thank you both for your time, your wisdom and your patience. It has been an absolute pleasure to get to know you and an honor to work with you.

The Cancer Biology PhD program and the Cancer Network Zurich have introduced me to many people that I sincerely hope will remain a part of my life, even though our path may soon split. I have especially grown close to Daniel Stiehl, MD, Department of Physiology, soon to be Dr. Ossia Eichhoff and Msc. Caroline Hyde, both at the Department of Dermatology, University Hospital Zurich, who have become dear friends of mine and valued peers. Thank you for your support, scientific discussions and critical readings of any written material that has left my hand.

With my PhD position came a new world. The Institute of Molecular Cancer Research is an outstanding institution to work at. The infrastructure is excellent, although several Thursday morning meeting may witness else how. However, truth be spoken, the institute is well organize and well run! To that I can add the awesome crew consisting of people literally representing the world. Thank you all for being here, being you and being my friend.

I will save my acknowledgement of my wonderful family for my wonderful family. I will just mention, that there are two people, who are direct reasons for me to be where I am. My brother, Dr. Dipl. Med. Jesper Bonde and my partner in life, Markus Billeter. Without you two, my life would not have made me pass through the Institute of Molecular Cancer Research, Zurich, Switzerland.

Thank you!

MSc. Bonde, Anne-Katrine

Curriculum Vitae

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Research Interests:

Translational cancer research; specifically the identification and validation of diagnostic markers and novel cancer drug targets, development and implementation of accurate diagnostic tools and novel therapeutic approaches and development of tools to serve as basis for personalized treatments.

Employment:

December 2006- Sept. 2010	**Post Graduate Student,** Institute of Molecular Cancer Research, University of Zurich, Switzerland
Jan. 2005 - Dec. 2006	**Research Technologist I**, Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL, USA
Sept. 2003	**Scientific Supervisor**, Public Natural Science Program, University of Aarhus, Denmark
Aug. 2003 - Jan. 2004	**Student Employee** at Institute of Molecular Biology, Aarhus University, Denmark

Education:

December 2006- Aug. 2010 (Expected)	**Cancer Biology PhD Program**, Life Science Graduate School Zurich, University of Zurich & ETH, Switzerland.
2002 - 2004	**Masters Degree in Biology**, University of Aarhus, Denmark
1998 - 2002	**Bachelors Degree in Biology**, University of Aarhus, Denmark
1995-1998	**Secondary School Leaving Exam** (High school), Science major, Ribe Katedralskole, Ribe, Denmark

Scientific Projects

Dec. 2006 - Sept. 2010 **Doctorate Dissertation:** ***A Role for Tumor Associated Macrophages in Tumor Cell Invasion.***
The dissertation is based on data obtained from a 3.5 year full time laboratory study executed at the Institute of Molecular Cancer Research, University of Zurich, Switzerland. ***Manuscript submitted***

Jan. 2005 – Dec. 2006 **Research Technologist:**

1) ***Establishing Model Systems to Examine Mechanisms underlying RNA Interference and the miRNA Pathway***.
2) ***Functional Characterization of the JAB1/MPN domain in the Essential Splicing Factor Prp8.***

Aug. 2002 – June 2004 **Masters Thesis:** ***Functional Examinations of the Mammalian Nuclear Exosome – mRNA survaillance***. A thesis based on results obtained from a two year full time laboratory study in the Laboratory of Nuclear Biogenesis of mRNP, University of Aarhus, Denmark

MSc. Bonde, Anne-Katrine

Conceptual Expertise:

Cancer biology:	Tumor biology, tumor immunology, inflammation, tumor angiogenesis, tumor cell invasion & metastatic dissemination, tumor cell-tumor stroma signaling, tumor pathology, tumor genetics
Basic research:	Tumor genetics, genetic instability, RNA processing & survaillance, RNA interference, miRNA pathway

Experimental & Technical Expertise:

In vivo tumor models	**Murine syngeneic tumor models**
In vitro models	**Mammalian cancer cell lines**, S. cereviciaea as model system
Drug assessment	**Naked drug- and liposome-encapsulated drug administration,** intravenous and intraperitoneal administration *in vivo*, cytotoxicity assays *in vitro*
In vivo imaging tech.	***In vivo* imaging systems** (IVIS 200)
In situ imaging tech.	**Fluorescence-, confocal- and light microscopy**
Cell biology	**Classical cell culturing** (details upon request)
Tumor pathology	**Tissue histology, immunohistochemistry** (tissue and cell based)
Biochem. & Mol Biol	**Standard RNA, DNA and protein technology** (details upon request), RNAi & miRNA technology *in situ*, *in vitro* and *in vivo*

Recent presentations at Symposia and International Conferences:

Oral:

Oct. 2009	**Guest speaker, Seminars in Applied Cancer Research**, Department of Radio-Oncology, University Hospital Zurich, Switzerland
April 2009	**2nd Cancer Biology Student Retreat**, Cancer Biology PhD Program, University of Zurich & ETH, Switzerland
April 2008	**Guest speaker, Seminars in Applied Cancer Research**, Department of Radio-Oncology, University Hospital Zurich, Switzerland,
Jan. 2008	**3rd Cancer Network Zurich Retreat,** Cancer Network Zurich, Switzerland
Sept. 2007	**1st Cancer Biology PhD Student Retreat**, Cancer Biology PhD Program, University of Zurich & ETH, Switzerland

Poster:

Oct. 2009	**4th Cancer Network Zurich Retreat**, Cancer Network Zurich, Switzerland
June 2009	**12th Beatson International Cancer Conference,** *Microenvironment, Motility and Metastasis*, Glasgow, Scotland
Feb. 2009	**9th Charles Rodolphe Brupbacher Symposium**, *Targets for Cancer Prevention and Therapy*, Zurich, Switzerland
Oct. 2008	**20th EORTC-NCI-AACR Symposium,** *Molecular Targets and Cancer Therapeutics*, Geneva, Switzerland
Jan. 2008	**4th Swiss Experimental Surgery Symposium,** *Non-invasive Cellular & Molecular Imaging in Small Animal Models*, University of Geneva and University Hospital Geneva, Switzerland

Additional training:

June 2008	**LightCycler Workshop 2008,** Roche Diagnostics, Rotkreuz, Switzerland. Advanced Training for Efficient Quantification and Genotyping on Roche LightCycler Instruments
Aug. 2007	**Animal Experiment Course,** Module 1, University of Zurich, Switzerland. Certified for animal experimentation, including training on ethical aspects.
June - July 2007	**Imaging Techniques,** Laboratory of Angiogenesis, Professor Gou Young Koh, Advanced Institute of Science and Technology (KAIST), Daejeon, Korea.

Additional activities:

Oct. 2009	**Teachers Assistant,** Student Course (Maters) in Applied Molecular Cancer Research, University of Zurich & ETH, Switzerland.
Nov. 2008 - April 2009	**Co-organizer of the 2nd Cancer Biology Student Retreat**, Cancer Biology PhD Program, University of Zurich & ETH, Switzerland.
March - July 2006:	**Assisting the Office of Research Safety** in developing a research safety educational program for students enrolled in PhD programs, Northwestern University, Evanston, IL, USA

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Jan. 2008	**First prize poster award**, 4th Swiss Experimental Surgery Symposium, *Non-invasive Cellular & Molecular Imaging in Small Animal Models*, University of Geneva and University Hospital Geneva, Switzerland.
July 2006	**Received special recognition** for the assistance in developing a research safety educational program for students enrolled in PhD programs, Northwestern University, Evanston, IL, USA

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German (Advanced knowledge)
Norwegian
French (Basic knowledge)

About me:

My passion for cancer research originates from a genuine curiosity towards cancer's complex biology, and a sincere wish to develop products of benefit to patients in need. Even after three decades of rapid advances in understanding, diagnosing and treating cancer, the disease remains the second largest cause of death in the western world. This highlights the need for continued efforts to improve the general understanding of its biology and develop novel tools for early detection and optimized indivudual treatment plans. I believe that joint efforts by experts from the various cancer research disciplines are the best and quickest way to develop these tools. I personally hope to contribute to these efforts through my solid understanding of the biological mechanisms driving cancer, while using my creativity and discipline to bring ideas from the drawing board to reality.

Other interests:

Outdoor sports, music, literature, art including own efforts in photography and painting

References:

Upon request